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CYTOCHROME OXIDASE IN ANTERIOR AND POSTERIOR BLASTOMERES OF CIONA INTESTINALIS

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The cytochemical test for indophenol blue oxidase, the indophenol or "nadi" reaction, has long been used to demonstrate supposed gradients or localizations of this enzyme in a wide variety of embryonic material. A particularly striking localization of this enzyme, presumably cytochrome oxidase, has been claimed for the ascidian egg where the myoplasm, presumptive for the larval musculature, gives an intense reaction with the indophenol reagents (Ries, 1937, 1939; Reverberi and Pitotti, 1939).

Cytochemical tests for demonstrating localization of enzymes in living cells are open to serious criticisms. Differential rates of staining of cells or of areas of a cell could be due to a variety of factors other than differences in enzyme activity, and it is extremely difficult to evaluate the sources of error. The aim of the present investigation was to make quantitative measurements of cytochrome oxidase in the ascidian blastomeres which stained differentially with the indophenol reagents. At the four-cell stage, the ascidian embryo consists of anterior and posterior blastomeres, the latter giving a strong oxidase reaction. Techniques were developed for separating the two types of blastomeres in sufficient numbers for quantitative analyses of cytochrome oxidase by a microspectrophotometric method. The results reported below verify the conclusions reached by previous investigators using cytochemical methods.

METHODS

Gametes were obtained from *Ciona intestinalis* by removing the test and opening the oviduct and vas deferens. Chorions of the unfertilized eggs were digested off with a protease solution (3% crude protease[1] in sea water) and the "naked" eggs were washed, transferred to agar-coated dishes and fertilized. An agar coat on all glassware is necessary to prevent sticking and subsequent cytolysis of the eggs. Several hundred first-cleavage blastomeres were separated from one another and in turn segregated by means of a breaking pipette from the remaining mass of eggs. Division of each isolated first-cleavage blastomere resulted in an anterior and posterior cell which were separated with the tip of a breaking pipette and transferred to separate dishes. Although they are identical in volume the anterior and posterior blastomeres can be distinguished by the elongated shape and clear cytoplasmic "cap" of the posterior blastomere (Castle, 1896). These characteristics are temporary and identification and separation of the blastomeres must be performed rapidly.

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[1] Purchased from Worthington Biochemical Sales Co., Freehold, New Jersey.

Cytochrome oxidase was measured by a microspectrophotometric method originally described by Cooperstein and Lazarow (1950) and repetitiously by Hess and Pope (1953). Basically, the method consists of the spectrophotometric observation of the rate of oxidation of reduced cytochrome *c* with the Beckman spectrophotometer adapted for the use of microcuvettes (Lowry and Bessey, 1946).

In more detail, the method as used for determination of cytochrome oxidase in eggs is as follows. The eggs or blastomeres were transferred by means of a breaking pipette to a 0.1-ml. conical-shaped centrifuge tube. By light centrifugation the cells were forced to the end of the tube and excess sea water was then drawn off with a pipette. Four to five μl. of homogenization medium were added, and the eggs homogenized by drawing them in and out of a fine pipette having a bore less than the diameter of an egg. The homogenization medium consisted of 0.75–1.0% sodium desoxycholate in phosphate buffer. This has been shown to have a clarifying action on crude homogenates and apparently causes a dispersal of the cytochrome oxidase complex (Hess and Pope, 1953). An attempt was made to reduce deterioration of cytochrome oxidase after homogenization by including serum protein in the homogenization medium (Borei, 1950). Although with some types of material the added protein seemed to offer some protection, its inclusion was not essential in the present experiments. Loss of enzyme activity was reduced considerably by rapid homogenization of the eggs at 3 to 4° C. Temperature control was obtained by placing the homogenization tube in a previously chilled copper block with drilled holes for microscopic observation.

The above procedure required only 20 to 30 seconds and the homogenate was then added to 45 μl. of reduced cytochrome *c* solution (1.6×10^{-5} M cytochrome *c*, reduced with sodium hydrosulfite, in 0.04 M phosphate buffer, pH 7.2) contained in a 50-μl. microcuvette. The reactants were stirred with a fine glass rod and the change in optical density at 550 millimicrons was followed for 2.5 minutes in a Beckman spectrophotometer. At the end of this period a small amount of potassium ferricyanide was added and the optical density of completely oxidized cytochrome *c* was measured. The measurements were carried out at room temperature (22 to 24° C.).

With the concentration of reactants used the reaction was first order and cytochrome oxidase activity could be expressed as the velocity constant of the reaction. The optical density of oxidized cytochrome *c* was subtracted from the readings at the various time intervals and the logarithms of these differences were plotted against time. This gave a straight line from which a velocity constant $\frac{(\Delta \ \log \ \text{ferrocytochrome } c)}{\Delta \ T}$ could be calculated (Cooperstein and Lazarow, 1950). Blanks (cytochrome *c* + homogenization medium) were carried out with each experiment in order that a correction could be made for the slow autoxidation of reduced cytochrome *c*.

In the present experiments the interest is in relative enzyme activities; accordingly all results are expressed as velocity constants. As can be seen from the cali-

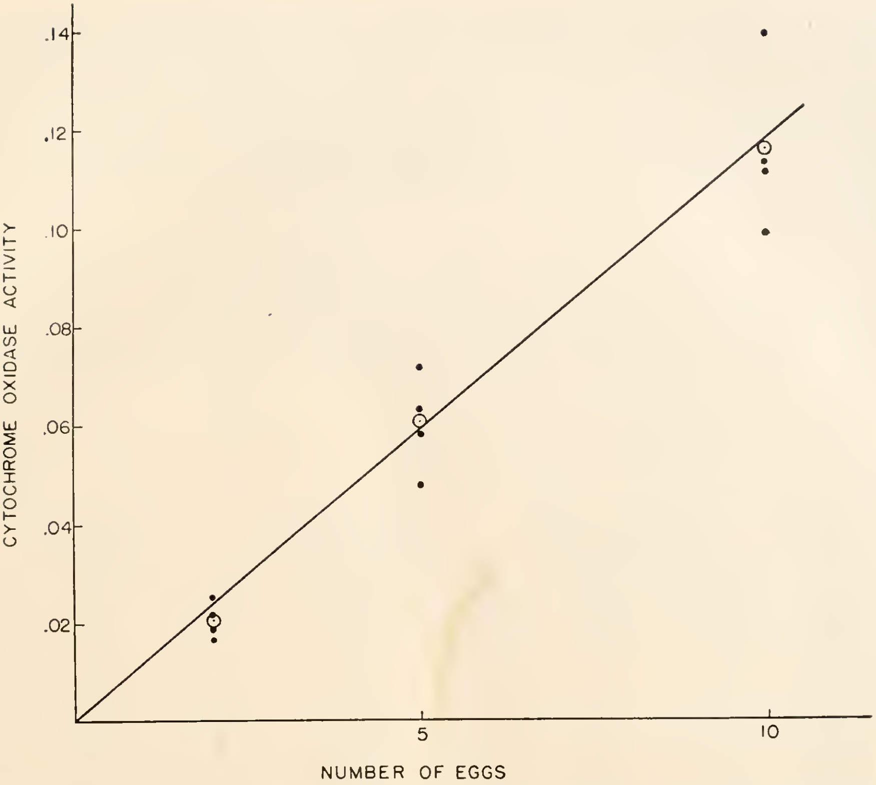

FIGURE 1. Cytochrome oxidase activity (Δ log ferrocytochrome *c*/min.) in homogenates of *Ciona* eggs. Solid points are individual measurements, large circles are averages.

bration curve (Fig. 1) these are proportional to the amount of cytochrome oxidase. The method is extremely sensitive, and it is possible to measure the cytochrome oxidase activity in a single *Ciona* egg.

RESULTS

Anterior and posterior blastomeres of *Ciona* were separated, and the cytochrome oxidase activities measured with the above described method. From previous work in determining the calibration curve it was decided that a sample containing about 20 second-cleavage blastomeres provided sufficient cytoplasm for reasonably accurate measurement of enzyme activity. In the first experiments 15 to 20 each of the anterior and posterior blastomeres were used, and the results were satisfactory. Straight lines representing reaction rates were obtained, and there was little subjective error in determining the slopes of the lines. As more skill in separating blastomeres was developed, the number used for each test was increased.

Several precautions were necessary in measuring the cytochrome oxidase of the blastomeres. A freshly prepared reduced cytochrome *c* solution tends to give high values for a standard cytochrome oxidase preparation (in these experiments, a standard number of whole, unfertilized eggs). As the preparation ages, over a period of several hours, the values for the standard decrease. For comparative measurements of enzyme activity this error can be eliminated by running the samples simultaneously using several cuvettes, or it can be corrected by alternately measuring single samples. The average of the values should then eliminate the error due to a change of the stock cytochrome *c* solution. The latter method was chosen as it is more convenient and permits better standardization of the homogenization procedure.

In all experiments the freshly prepared cytochrome *c* was tested several times with whole eggs before it was used with the isolated blastomeres. The time between determinations of the enzyme in the two types of blastomeres was shortened by carrying the experimental procedure for both types of blastomeres up to the point of homogenization. The results, tabulated in Table I, are expressed as velocity constants which are proportional to cytochrome oxidase activity (Fig. 1).

TABLE I

Cytochrome oxidase activity in homogenates of anterior and posterior blastomeres of Ciona

Number of blastomeres	Cytochrome oxidase (10^2 Δ log ferrocytochrome *c*/min.) in anterior blastomeres	Cytochrome oxidase (10^2 Δ log ferrocytochrome *c*/min.) in posterior blastomeres	Ratio cytochrome oxidase activities $\left(\frac{\text{post.}}{\text{ant.}}\right)$
20	8.0	14.8	1.9
15	2.0	4.2	2.1
24	5.4	15.3	2.8
24	4.7	11.3	2.4
27	2.4	10.7	4.5
32	4.1	14.5	3.5
36	5.9	10.9	1.9
32	5.0	15.3	3.1
32	7.8	14.0	1.8
			average 2.7

Since the majority of experiments were carried out under varied conditions (different batches of eggs, cytochrome *c* solutions, temperatures, etc.) it is not possible to compare velocity constants of separate experiments with any degree of reliability. The data in Table I must therefore be considered as a paired series with the results best expressed as ratios (shown in the last column of Table I). The cytochrome oxidase activity in posterior blastomeres averages 2.7 times that in anterior blastomeres, a difference which is sufficiently great to eliminate the need for a statistical test for significant difference. These results cannot be due to volume differences since the second cleavage is an equal one. The average diameters, as measured with an ocular micrometer, of 75 anterior and 91 posterior blastomeres were 103 microns and 104 microns, respectively.

DISCUSSION

As shown in Table I, the ratios in enzyme activity between the blastomeres vary from 1.8 to 4.5. To what extent this range of values reflects experimental error is difficult to determine. The data in Figure 1 give some indication of accuracy although not all these measurements were made consecutively. A test for reliability of the method gave very good results; thus, in a series of determinations using five eggs each time, the velocity constants were 0.083, 0.074, 0.073, 0.083, 0.076. Ultramicro methods are particularly subject to erratic and inexplicable errors, and the present method is no exception. One very probable source of error is the homogenization procedure which, due to the very small amounts of material, is difficult to standardize. These and other errors, however, tend to be eliminated by averaging the results of separate experiments, and it is felt that the average value in Table I is reasonably accurate.

These results have considerable value in support of the cytochemical studies which have been carried out on the ascidian egg. Ries (1937, 1939), Reverberi and Pitotti (1939), Urbani and Mistruzzi (1947), using the indophenol reaction, demonstrated a localization of indophenol blue oxidase (presumably cytochrome oxidase) in posterior blastomeres of various ascidian embryos. The present results are free from the objections which may be raised against cytochemical techniques; however, as it turns out, they offer a striking confirmation of the earlier cytochemical results.

The indophenol reaction has also been used with other types of eggs and embryos, principally by Child (1941) and his students. Thus differential appearance of color with the reagents has been demonstrated in sea urchin, amphibian, and chick embryos. The results presented here indicate that the use of this cytochemical test is valid, and that localization or gradients of cytochrome oxidase probably exist in these other forms.

As is known from studies on other types of cells, the cytochrome oxidase complex is located in mitochondria and presumably this is true in the *Ciona* egg. The localization of cytochrome oxidase in the particulate fraction of centrifuged homogenates of *Ciona* eggs and the action of sodium desoxycholate on crude homogenates lend support to this assumption. The results, therefore, indicate a marked localization of mitochondria in the posterior blastomeres.

From cell lineage studies of the ascidian egg it is known that the myoplasm, the region rich in cytochrome oxidase, gives rise to the muscles of the tadpole. Conklin (1931) demonstrated that displacement of the pigmented inclusions from the myoplasm of *Styela* eggs did not alter subsequent differentiation of muscles. Similarly located "mitochondria" in *Ciona* eggs were not essential for muscle formation. Although Conklin refers to these inclusions as mitochondria, he reserves the possibility that they may be lipid granules due to their staining properties and their low density as indicated by displacement to the centripetal end of centrifuged eggs.

There are indications that these granules lack cytochrome oxidase which is an additional reason for not referring to them as mitochondria. In centrifuged eggs the indophenol reaction is absent in the centripetal region (Ries, 1939; Urbani and Mistruzzi, 1947). Experiments carried out in this laboratory demonstrate that the cytochrome oxidase-containing elements have a high density. Egg homogenates (50 eggs in 5 μl. phosphate buffer) were fractionated by centrifugation and the cytochrome oxidase measured by the microspectrophotometric method. The light fraction composed of oil droplets and small granules, presumably the "mitochondria" described by Conklin, contained little or no cytochrome oxidase whereas the heavier fraction contained nearly all the enzyme. In another experiment a solid mass of eggs (about 5 μl.) with no excess fluid was homogenized and fractionated by centrifugation. Samples of the light fraction consisting of oil droplets and small granules showed little cytochrome oxidase activity as compared to a high oxidase activity of the heavier fractions.

It seems reasonable to conclude that the inclusions which Conklin described in the myoplasm of the ascidian egg are not cytochrome oxidase-containing mitochondria and therefore their lack of influence on muscle differentiation has no bearing on whether a high oxidase activity is necessary for such differentiation.

Experiments which are more pertinent to this question are those of Ries (1939) who concluded that the cytochemically demonstrated oxidases were essential for normal differentiation of muscles. The region of the centrifuged egg which gave rise to muscles always coincided with that which gave a strong oxidase reaction. Furthermore if the reactive cytoplasm was displaced by centrifugation, the muscles differentiated in an abnormal region. In addition Reverberi and Pitotti (1939) relate the presence of the oxidases to normal cleavage and development of bilateral symmetry.

These authors, quite rightly, do not consider their experiments as indicating that oxidases are the primary cause of differentiation. It seems obvious that if there is a segregation during cleavage of particulate elements such as mitochondria, we cannot assume that our indicator of this segregation, such as cytochrome oxidase, is in itself the cause of differentiation. Particulate elements are complex in structure and function, all of which factors would have to be considered with respect to differentiation rather than a single component.

Mitochondria are generally considered to be the "power plants" of the cell and supply energy for cellular activities. The potential for energy production of posterior blastomeres might be related to subsequent differentiation, although at an early stage their energy requirement, as measured by oxygen consumption, cannot be more than 20% different from that of anterior blastomeres (Holter and Zeuthen, 1944). It is evident that more information is needed before we can be specific in relating differentiation to a localization of specific enzymes, or even, as the results suggest, a differential distribution of mitochondria.

SUMMARY

Anterior and posterior blastomeres were separated in the four-cell stage of *Ciona intestinalis* and cytochrome oxidase activities of homogenates of the blastomeres were quantitatively determined by a microspectrophotometric method. The average cytochrome oxidase activity of homogenates of posterior blastomeres is 2.7 times that of anterior blastomeres. The results indicate a localization of mitochondria in the posterior blastomeres.

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THE FAILURE OF HYPOPHYSECTOMIZED FUNDULUS HETEROCLITUS TO SURVIVE IN FRESH WATER

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A pituitary control of osmoregulation in *Fundulus heteroclitus* was first suggested by Pickford (1953). She noted that six hypophysectomized fish which had been placed in fresh water were unable to survive, although such operated fish would live indefinitely in sea water and normal *Fundulus* will withstand abrupt changes of salinity with no adverse effects. These results were unexpected, since hypophysectomized *F. heteroclitus* (Matthews, 1933; Abramowitz, 1937), as well as similarly operated eels (Fontaine *et al.*, 1949; Callamand *et al.*, 1951), and certain strictly fresh water teleosts (*e.g.*, Abramowitz, 1937; Chavin, 1954) have been reported to survive in fresh water for considerable periods of time.

The problem of osmoregulation in teleostean fishes has been extensively studied and is reviewed by Baldwin (1937), Krogh (1939), Black (1951) and Fontaine (1953). Despite much research, a hormonal control of water and salt balance, such as is known in higher vertebrates, has not been conclusively demonstrated in teleosts. In the mammal, pituitary control of osmoregulation involves at least two antagonistic renal functions: the neurohypophyseal antidiuretic factor (vasopressin) acting directly on the kidney tubules (reviewed by Gaunt *et al.*, 1951) and the growth hormone (GH) acting apparently in conjunction with corticotropin (ACTH) and thyrotropin (TSH) through the adrenal cortex, to increase urine flow (reviewed by White, 1955). In amphibians the neurohypophysis regulates water metabolism by both renal and extrarenal mechanisms. Injections of neurohypophyseal hormones promote glomerular antidiuresis, and resorption of water by the kidney tubules and bladder (Sawyer, 1956). Water uptake, which takes place primarily through the skin, is increased by the injection of neurohypophyseal hormones. This response is diminished after hypophysectomy but may be restored to normal by a combination of ACTH and TSH (Levinsky and Sawyer, 1952).

Mammalian neurohypophyseal hormones appear to have no effect upon teleostean osmoregulation (Keys and Bateman, 1932; Burgess, Harvey and Marshall, 1933; Boyd and Dingwall, 1939; Fontaine and Raffy, 1950). Salmon posterior lobe is also ineffective (Callamand *et al.*, 1951). Nevertheless, Arvy *et al.* (1954, 1955) observed that there was a depletion of neurosecretion in the hypothalamo-hypophyseal system of *Phoxinus* and *Anguilla* after transfer to hypertonic media. In mammals it is well known that an increased salt load leads to a depletion of neurosecretory material, correlated with a decrease in the content of antidiuretic

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hormone in the hypothalamo-hypophyseal system (see Bargmann, 1954). The secretion of neurohypophyseal hormones in mammals apparently also stimulates the rapid release of corticotropin (Martini and Morpurgo, 1955) and thyrotropin (D'Angelo, 1955) in response to stress. Therefore the depletion following salt stress in fish does not necessarily signify an antidiuretic function of the neurohypophyseal hormone(s).

However, there is some evidence of hormonal control of water-electrolyte regulation in fish. Kalashnikov and Skadovskii (1948) observed lowered blood chloride and osmotic pressure following injections of hypophyseal extracts in the sturgeon. Smith (1956) found that salinity tolerance was increased in trout by treatment with growth hormone. Many investigators have presented evidence favoring greater thyroid activity in hypotonic than in hypertonic media in a number of teleostean species; this was recently demonstrated, for example, in *Fundulus* by Gorbman and Berg (1955), using radioactive iodine uptake studies. Fontaine (1956) notes that survival of a number of marine teleosts in fresh water may be significantly prolonged by administration of thyroxin or TSH before transfer. Chavin (1954) found a transitory stimulation of the adrenal cortical tissue of goldfish following saline immersion which he attributed to release of ACTH. Keys and Bateman (1932) showed that adrenalin enhances chloride secretion in the eel gill, but the relation of adrenalin secretion to the pituitary is not obvious.

Regardless of the hormones which may be involved, three sites of salt and water balance in teleosts may be affected. Although fish lack the dermal water balance mechanism found in amphibia, hypophysectomy could affect the permeability of the water barrier, thereby having a profound effect upon survival in non-isotonic media. A second site is obviously the kidney, which is actively concerned in water elimination in fresh water fish. Henschel (1934–35) found that the euryhaline plaice, but not the stenohaline sole, was able to increase urine flow upon transfer to fresh water. But it must be noted that on the basis of morphological studies Grafflin (1937) concluded that the failure of fresh water teleosts to adapt to sea water or *vice versa* must be attributed to extrarenal factors.

The salt-regulating function of the gills is well known. Considerable attention has been given to the cytological aspects of this mechanism. Chloride excretion was first attributed to large, acidophilic cells in the gill epithelium by Keys and Wilmer (1932). These "chloride cells" were found to be absent in the dogfish but abundant in six marine species of teleosts. In four fresh water forms the cells were infrequent or absent although acidophilic cells lacking contact with both the surface and the basement membrane were noted. Mucous cells were more abundant in the latter group.

Liu (1942) demonstrated that an enormous development of "chloride cells," previously hardly distinguishable in the gill of the paradise fish, *Macropodus opercularis,* could be brought about by gradually increasing the salt concentration of its environment. Copeland (1948), investigating the "chloride cells" of *Fundulus,* found that they characteristically possessed an open, distal vesicle in the sea water-adapted state which was lost, a clear protoplasmic area being found in its place, when the fish were transferred to fresh water. The critical salinity separating the salt and fresh water pictures was thought to lie between $\frac{1}{16}$ sea water and $\frac{1}{100}$ cold-blooded Ringers solution in tap water (Copeland, 1950).

Getman (1950) found the same cell type in *Anguilla rostrata* with far less marked cytological variation between fish adapted to fresh or salt water. He found that the vesicles or "pits" were less frequent, not absent, in fresh water adaptation, but noted that the results might be attributable to experimental conditions. Hoar (1951) mentioned the development of "chloride cells" at the time of parr-smolt transformation in the Atlantic salmon preparatory to seaward migration. Nishida (1953) found that "acidophilic great cells," which he identified with the "chloride cells" of Hoar and Copeland, were much more abundant and better developed in the smolt of the sea-run than the land-locked variety of *Onchorynchus masou* while both were still in fresh water.

The present investigation was concerned with a number of endocrinological and histological aspects of the problem in *Fundulus*. After confirmation of Pickford's findings regarding the loss of ability of hypophysectomized sea water-adapted *F. heteroclitus* to live in fresh water, hypophysectomy of fresh water-adapted fish was used to determine whether the impairment is that of survival or adaptation. Replacement therapy was employed to verify the fact that the observed condition is due to pituitary loss rather than to another factor inherent in extirpation of the gland, and an attempt was made to identify the pituitary fraction which enables the animal to live. In investigating the cause of death, studies of weight and blood chloride changes were made. Lastly, an examination of the gill cytology of the fish was carried out in search of clearly defined histological differences which might help explain the osmoregulatory difficulties.

I wish to express my sincere appreciation to Dr. Grace E. Pickford for suggesting the problem and for her guidance and encouragement. For making available the facilities of the Bingham Oceanographic Laboratory for these investigations I owe my thanks to its director, Dr. Daniel Merriman. I am indebted to other members of the laboratory staff; to Dr. Gordon A. Riley for determining water salinities, to Mr. Edward C. Migdalski for supplying the perch, and to the Misses Harriet A. Chambers, Rita M. Eurich, and Patricia J. Harris for their technical assistance and advice. I also thank Dr. Sanford L. Steelman of Armour and Company for the ACTH, TSH, and posterior lobe preparation, Dr. A. E. Wilhelmi at the Department of Biochemistry, Emory University, for the purified growth hormone, Dr. Tillman D. Gerlough of E. R. Squibb and Sons for the thyroxin, and Dr. Richard H. Barnes of Sharp and Dohme for the desoxycorticosterone acetate. Facilities for collecting sea water were provided by Dr. Victor L. Loosanoff, director of the U. S. Fish and Wildlife Service Laboratory at Milford, Connecticut. Finally, for translations of the Russian and Japanese literature employed I express my appreciation to Dr. Alexander Petrunkevitch and Mr. Mikiso Hane, respectively.

MATERIALS AND METHODS

1. Running fresh water system

In order to test a relatively large number of hypophysectomized fish and controls under satisfactory conditions a running fresh water system was required. Such a system was necessary to avoid possible adverse effects from the handling involved in daily water changes or the definitely undesirable salt accumulation which would occur in a standing tank from addition of food, evaporation, etc. As the only fresh water readily available was from the municipal water supply, three problems presented themselves in designing the required system: constant rate of flow, constant temperature, and dechlorination.

The arrangement is shown in Figure 1. Tap water was received into a constant level tank (A) placed above the aquaria. The water flowed from the tank by gravity at a rate regulated by a clamp (B) through a series of ten five-gallon carboys (C 1–10). The first two contained activated charcoal in sausage skins (D 1 and 2) to remove dissolved chlorine (modified method of Coventry, Shelford and Miller, 1935). The outflow from the last carboy was dripped into an aerated container (E) for the purpose of reoxygenation. From here water was fed into the aquaria (G 1 and 2) by means of a constant level siphon (F 1), a similar arrangement being used to adjust the outflow (F 2).

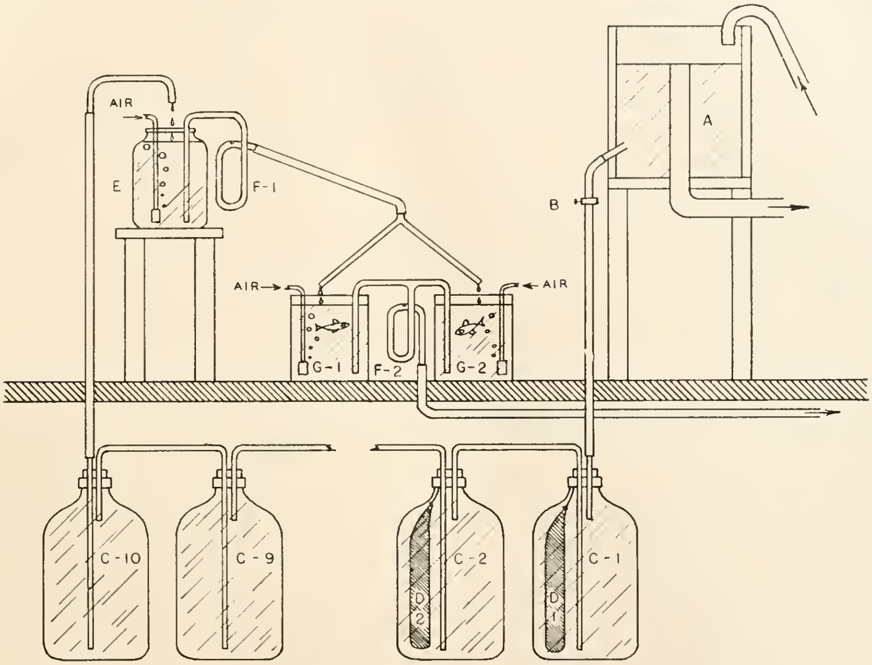

Figure 1. Running fresh water system (drawn by Patricia J. Harris).

The flow of water was regulated to deliver ten gallons a day to each of two one-gallon aquaria, thereby giving a turnover rate of about once in every two and one-half hours. The battery of carboys created a reservoir such that it took over two days for water to pass through the system. During this time it reached aquarium room temperature which was regulated at *ca.* 15° C. Tests of the outflow with orthotolidine indicator showed it always to be chlorine-free. The only difficulty encountered was bubbles in the reservoir lines brought about by warming of the water. Flow irregularities due to these were easily avoided by removal of one of the carboy stoppers for a few seconds once or twice daily. Five to seven fish could be maintained comfortably in each aquarium.

2. *Experimental procedures*

All fish used were *Fundulus heteroclitus* males and females 2¾ to 3¼ inches in length. Hypophysectomy, feeding, and illumination arrangements were the same as described by Pickford (1953). Operations and injections were made under anesthesia with tricaine methane sulfonate. Fresh rather than salt water was used exclusively when such procedures were being carried out with fish being maintained in the fresh water system. All experiments were made at 14–17° C. The pH of the sea water from the storage tank and in freshly diluted mixtures was *ca.* 8.5. However, in long established sea water tanks where the fish were usually held it fell to *ca.* 7.4. The pH of the fresh water ranged from 7.2 to 7.6.

One or two unoperated controls were kept in the aquaria with each experimental group, making a total of about 30 normal fish being maintained in fresh water for periods of two weeks to three months each. All of these fish remained healthy, although, as also in the case of fish in salt water, it was occasionally necessary to treat them for minor skin infections, either with aureomycin or malachite green as recommended by Pickford (1954a). Hypophysectomized fish in salt water rarely die as an immediate result of the operation but a small number of fish, of the order of 5–10%, were lost at later dates from causes other than the experimental treatments. In all a total of about 100 hypophysectomized fish were employed in the experiments.

(*a*) *Tests on survival of "long term" hypophysectomized sea water-adapted fish in fresh water.* A group of about 60 fish that had been kept in sea water were hypophysectomized and returned to this medium. They were allowed to recover for at least two weeks and were then tested for their ability to survive in fresh water. Out of 56 so tested only three were able to live indefinitely, and these showed positive evidence of incomplete hypophysectomy (growth in length and, in males, development of nuptial coloration); they were therefore excluded from the experiment. The remaining 53 fish developed symptoms of asthenia, described below, but 39 of them were saved by gradual return to sea water through $\frac{1}{10}$, ¼, and ½ dilutions over a period of about 24 hours. Fish that recovered were kept under observation until they were again eating normally and then were allowed at least a few extra days before being used in other experiments. The average time for complete recovery was about two weeks.

(*b*) *Tests on survival of "long term" hypophysectomized sea water-adapted fish in diluted sea water.* These experiments were limited to a small number of previously untested fish and are of a preliminary nature. The tests were carried out in similar aquaria to those used in the fresh water system, the water being changed two or three times weekly. Two hypophysectomized fish and one unoperated control were tested in $\frac{1}{10}$ and ¼ sea water (*ca.* 2.6 and 6.5 ‰, respectively); four hypophysectomized fish and one control were tested in ½ sea water (*ca.* 13 ‰).

(*c*) *Tests on ability of fresh water-adapted fish to survive hypophysectomy in fresh water.* A group of 25 fish were kept in fresh water for about four weeks before operation and were returned to this medium after removal of the pituitary. Survival times were studied as in the preceding groups and those that recovered in sea water were saved for other experiments.

(*d*) *Tests on ability of sea water-adapted fish to survive transfer to fresh water immediately after hypophysectomy.* A small group of eight sea water-adapted fish were transferred to fresh water immediately after hypophysectomy. These served as controls for the group that had been pre-adapted to fresh water.

(*e*) *Weight changes in fresh and salt water.* Two series of experiments were made in each medium with four previously untested, hypophysectomized fish in each group. One series was weighed daily until onset of asthenia in the fresh water group and for a comparable time in the sea water group. A second series was treated similarly but received only initial and final weighings. Two unoperated controls were included with each group, except those receiving initial and final weighings in sea water, making a total of 22 fish employed in the experiment. Weighings were made on a triple beam balance following anesthesia and extremely light blotting. It is realized that the small numbers employed detract from the significance of this experiment but time did not permit further study and the results appear to be worth reporting.

(*f*) *Blood chloride determinations.* Blood chlorides were determined by a microadaptation of the method of Schales and Schales (Hawk, Oser and Summerson, 1947). Blood serum samples were collected from normal fish adapted to salt or fresh water (8 days) and from hypophysectomized fish adapted to sea water and on the verge of death in fresh water (4–7 days). Whole blood was obtained by cutting off the tail and allowing the blood to drop on to a paraffined spot plate. Ten- to 30-mm.^{3} samples were centrifuged in an air driven "spinning top" rotor (Glick, 1949) and 4.8-mm.^{3} quota of serum were transferred into titration vessels with the aid of a constriction pipette. Before titration two magnetic beads and *ca.* 45 mm.^{3} of water containing 0.06 ml. of indicator solution per 2 ml. were added to the sample. Titration was made without deproteinization using a 70-mm.^{3} microburette and magnetic stirrer.

(*g*) *Hormone therapy.* These experiments were all made on hypophysectomized fish that had been previously tested for their inability to survive in fresh water and had subsequently been allowed to recover in sea water.

Fourteen series of intraperitoneal injections were made in an attempt to determine what treatment would enable hypophysectomized *Fundulus* to survive in fresh water. Breis of *F. heteroclitus, Pollachius virens,* and *Perca flavescens* pituitaries, in addition to solutions or suspensions of ACTH, desoxycorticosterone acetate (DOCA), TSH, thyroxin, GH, posterior lobe preparation, a combination of ACTH, TSH, and GH, and three series of saline controls, were employed. The breis and hormones were made up in 0.6% NaCl. Unless otherwise noted, groups of four fish were given daily injections of 0.005 ml. per gram weight on alternate days with four injections (one week) in sea water before transfer to fresh water.

Fundulus pituitary glands were collected from fish weighing from seven to 40 grams, caught near New Haven just prior to spawning in April, 1955. Two hundred glands, averaging slightly under one milligram each in weight, were contained in each milliliter of homogenate so that each fish received one gland per gram weight at each injection. Fresh frozen glands from the stenohaline marine pollack were collected from prespawning fish during the summer of 1954, at Wilson's Beach, Campobello Island, by G. E. Pickford and assistants. The brei was prepared in about the same concentration by weight as the *Fundulus* material, 200 mg./ml. Two series of experiments were carried out using this preparation. The first was under the same conditions as with the *Fundulus* brei. The second series, comprising two hypophysectomized fish and one unoperated control, was given daily treatment with the homogenate at 0.01 ml. per gram and with but two injections before transfer to fresh water. Thus they received four times as much material as did the first series. The average weight of the pituitary of post-spawning yellow perch weighing from 30 to 100 grams each was found to be of the same order as that of pre-spawning *Fundulus* weighing 7–40 grams, *ca.* 1 mg., and hence the same concentration (200 glands/ml.) was used. Post-spawning fish, caught during June–July, 1955, in the New Haven area, were employed.

ACTH dosage (Armour's Acthar Lot No. R-491284-U, 0.05 USP units/gm.) was based on that used by Pickford (personal communication) who obtained definite stimulation (increased cell layers, proliferation) of the interrenals of *F. heteroclitus* within one week. Desoxycorticosterone acetate injections (Sharp and Dohme Lot HXVII-06-07-4-1, 25 μg/gm.) were equivalent to those given by Kirshenblatt (1952) to vy'un (*Misgurnus fossilis*) in which he was able to induce egg maturation and ovulation. On the basis of the thyroid stimulation findings of Pickford (1954b) 1 μg/gm. was selected for injections of TSH (Armour Lot No. 317–51, 1.2 USP/mg.). About 5 $mm.^3$ 0.1 N NaOH/ml. was required to bring this into solution. Since there is no established function in fish on which to base dosage, thyroxin (Squibb BT00080) was somewhat arbitrarily set at 2 μg per gram weight. It is of the same order as that used by many investigators on fish and has been shown to increase resistance to skin infections in *Fundulus* (Pickford, unpublished observations). Addition of 20 $mm.^3$ 0.1 N NaOH/ml. was required to dissolve the hormone. In these two series, which were made early in the investigation, before the effects of *Fundulus* brei had been established, only two injections in sea water prior to fresh water transfer were made and injections were given daily.

Growth hormone dosage (Wilhelmi Beef B168GH, 10 μg/gm.) was based on that given by Pickford (1954a) by which she obtained growth of the same order as occurs in normal fish. Ten $mm.^3$ 0.1 N NaOH/ml. were required for solution. Injections of whole posterior lobe preparation (Armour Hog Lot No. 503, 100 mU/gm.) were given at twice the dosage determined as one *Fundulus* unit in spawning reflex assays (Wilhelmi, Pickford and Sawyer, 1955). The concentration of each hormone in the GH-TSH-ACTH combination remained the same as when used separately: 10 USP units ACTH, 2 mg. TSH (Armour Lot No. 2R3, 0.4 USP/mg., less potent then 317–51 but still at a higher dosage than that used effectively by Pickford, 1954b), 10 mg. GH, 15 $mm.^3$ 0.1 N NaOH/ml. Of three series of saline controls, the first received injections of 0.6% NaCl under the usual conditions. A second and a third group, each with two hypophysectomized fish and an unoperated control, were injected daily with 0.6% and 0.06% NaCl. No build-up injections in sea water were made with these last two series.

(*h*) *Histological study.* The thyroids and the interrenals of the group receiving *Fundulus* brei were treated as previously described by Pickford (1953). Hemoglobin was determined with the aid of a Tallqvist-Adams scale. The study of the gill cytology was carried out according to the Regaud-Altmann mitochondrial technique recommended by Copeland (1948). Experience proved that best results could be obtained if the picric acid differentiating fluid were used merely to wash off excess acid fuchsin after cooling and differentiation were accomplished by flooding the slide with 1% aqueous methyl green for about 5–10 seconds. It is worth noting that the heating of Altmann-flooded slides should be stopped when white fumes are first evoked.

Gills of four normal sea water-adapted *Fundulus,* five normal fresh water-adapted specimens, six hypophysectomized fish which were sea water-adapted, four hypophysectomized *F. heteroclitus* on the point of death in fresh water, and those of the series receiving *Fundulus* brei were selected for study. Each set was also stained with hematoxylin and eosin and by the azan method for confirmatory studies.

RESULTS

(*a*). Of the total of 53 long-term hypophysectomized fish tested by transfer to fresh water, 14 were found dead or so nearly so as not to recover subsequently.

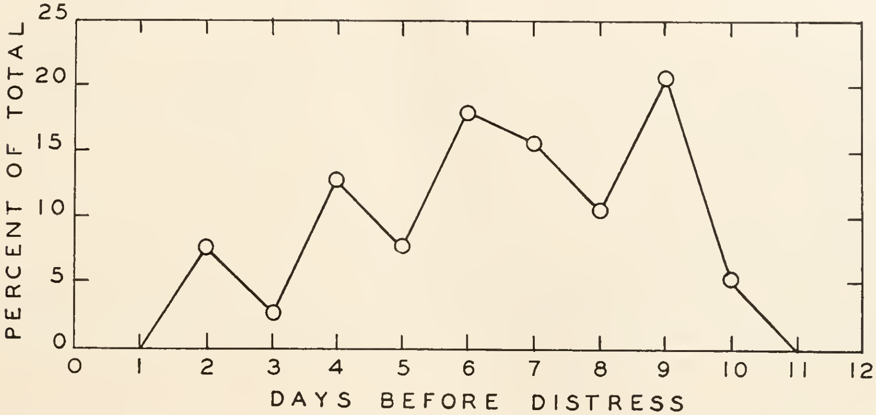

FIGURE 2. Time of onset of asthenia in 39 sea water-adapted "long term" hypophysectomized *F. heteroclitus* after transfer to fresh water. Only fish that were saved and recovered in sea water are used (see text).

The 39 remaining fish, removed when found lying on their sides, recovered completely after being transferred to sea water. These latter fish and the number of days they remained in fresh water before removal was necessary, are shown graphically in Figure 2. Only these 39 are considered since it is felt that subsequent recovery in sea water is the most conclusive demonstration that the fresh water was the sole cause of their distress.

The following symptoms preceded death in fresh water: The fish refused to eat after the first or, occasionally, the second day. After about two thirds of the total time which elapsed before death they became extremely quiescent and stayed on the bottom of the aquarium. About a day before death the fish were noticeably tilted and would be found lying completely on one side twelve hours later, usually ventilating their gills rather rapidly. This was the point at which there could be no doubt that the fish were succumbing and this stage was selected for rescue for use in subsequent experiments. Shortly before death they would be found completely upside down and frequently could be saved even in this state. Return to sea water was accompanied by hydrostatic and equilibrium difficulties far exceeding those experienced by normal fish.

TABLE I

Time of onset of severe asthenia in "long term" hypophysectomized, sea water-adapted F. heteroclitus in different dilutions of sea water

Per cent sea water	Number of fish	Days				
		5–10	11–15	16–20	21–25	26–30
10%	2	—	2	—	—	—
25	2	—	—	2	—	—
50	4	1	—	1	—	2

Time did not permit study of the ability of these fish to withstand a second transfer to fresh water, since the tested fish were needed for hormone therapy. Such a project would have required the study of the entire group, in order that possible differences in mean survival time would be statistically significant, and this would have greatly curtailed the study of other aspects of the problem. For replacement therapy experiments it appeared more important to use saline-injected controls since it is known that such treatment tends to increase the weight loss and lower the viability of hypophysectomized *F. heteroclitus* in salt water.

(*b*). Table I summarizes the results for fish tested in diluted sea water. At all three dilutions the normal controls were still active and eating long after their hypophysectomized counterparts had succumbed.

(*c and d*). The results obtained by operating on fresh water-adapted fish and placing sea water-adapted fish in fresh water immediately after the operation are graphed in Figure 3. Owing to the small number of cases in these two groups,

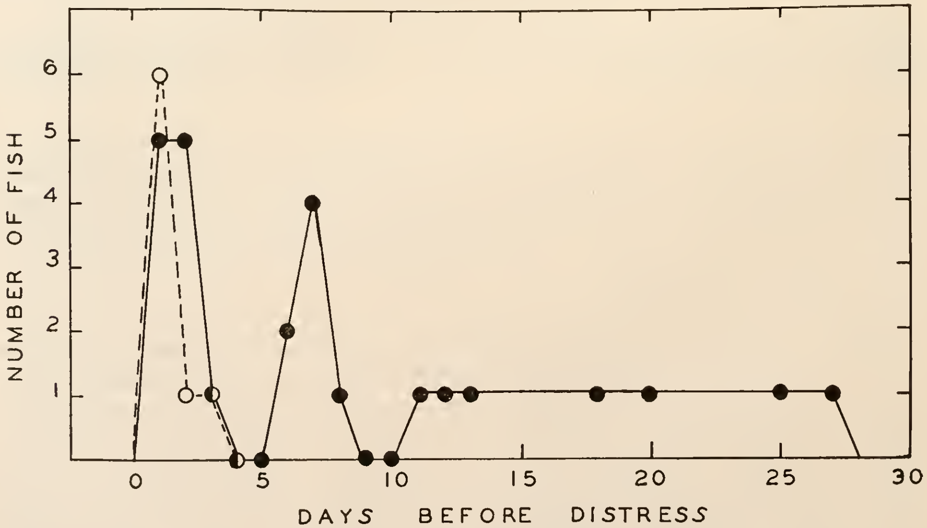

FIGURE 3. Time of onset of asthenia or death in hypophysectomized *F. heteroclitus* placed in fresh water immediately following the operation. Solid line represents 25 fish previously fresh water-adapted. Broken line indicates the eight previously sea water-adapted.

TABLE II

Weight change tests

Group		Days	No. of fish	Mean weight change	*s*
Daily weighings:					
Fresh water	Hypects	2–3	4	13.8	3.4
	Controls	2	2	−0.1	—
Sea water	Hypects	3	4	−4.0	3.2
	Controls	3	2	−6.5	—
Two weighings only:					
Fresh water	Hypects	6–9	4	4.3	4.5
	Controls	10	2	7.0	—
Sea water	Hypects	6	4	−4.1	5.0

those which did not recover subsequently are included as well. Out of the group of 25 fresh water-adapted fish, 11 succumbed during the first three days, seven survived 6–8 days, and another seven survived 11–27 days. In the second group, all died in the first three days, six after one day, and one on each of the following two days.

(*e*). Table II contains the results of the weight change tests. Hypophysectomized fish succumbed more rapidly in fresh water when handled daily than when weighed only at the beginning and end of the experiment, 2–3 days as compared with 6–9 for the latter group. Both hypophysectomized groups showed a slight weight increment at the time of onset of asthenia in fresh water, greater in the case of daily handling (13.8% versus 4.3%). Hypophysectomized fish in sea water showed a small decline (*ca.* 4%), irrespective of whether they were handled daily or not. The number of controls is too small in each group, but appears to indicate that daily handling causes weight loss in sea water but little change in fresh water. A mean weight increment in unhandled fish in fresh water is probably due to natural growth, since a period of 10 days elapsed.

(*f*). In Table III blood chloride determinations are given. Normal fish in either sea water or fresh water maintain a constant blood chloride level (0.885–0.887) and the blood chloride of hypophysectomized fish in sea water is not sig-

TABLE III

Blood chloride determinations

Group	Number of fish	Serum NaCl, gm.%		
		Range	Mean	*s*
F.W. Hypects	6*	.322–.451	.383	.049
F.W. Normals	6	.809–.996	.887	.078
S.W. Hypects	5	.777–.872	.817	.036
S.W. Normals	5	.818–.957	.885	.051

* Samples were taken at the onset of acute symptoms. Three of the fish in this group had been receiving GH-TSH-ACTH and DOCA injections.

nificantly lower ($P = 0.05–0.1$). When hypophysectomized fish are placed in fresh water, however, a blood chloride averaging only 43% of normal is found when asthenia begins.

(*g*). The results of injection therapy are found in Table IV. The fish's numbers are given so that it can be seen which ones were used in more than one series and their performance in each compared. Those receiving *Fundulus* brei were still active and eating after 20 days in fresh water. At that time it was decided that the preparation was undoubtedly enabling them to survive, so the fish were killed for autopsy. Numerous fish being tested for fresh water survival for the first time or receiving injections of other preparations succumbed during this 20-day period.

The results of the autopsy of the fish receiving *Fundulus* brei (Table V) indi-

TABLE IV

Results of injection therapy

Material injected	Fish number	Days in fresh water		Subsequent recovery completed (days)
		Previous test	With injections	
Fundulus brei†	4	7	20	(Discontinued)
	25	(*)	20	(Discontinued)
	55	6	20	(Discontinued)
	107	9	20	(Discontinued)
Perch brei†	68	10	6	Died next day
	58	8	8	3
	B108	(*)	16	Found dead
	G3	6	19	(Discontinued)
Pollock brei (Alternate days)	14	6	2	5
	77	9	3	3
	68	10	5	4
	58	8	7	3
Pollock brei (Daily)	12	4	4	Died 3 days later
	11	3	6	Died 2 days later
ACTH	76	6	3	Found dead
	44	(*)	5	4
	78	7	5	3
	32	(*)	5	3
DOCA	44	(*)	2	Found dead
	14	6	2	2
	61	7	2	Died 16 days later
	32	(*)	4	Found dead
	G25	10	5	Blood sample taken

* Fish tested by use in weight change test or hypophysectomized after fresh water adaptation and hence not comparable.

† In each of these groups a fifth fish died under anesthesia after the fourth (perch) or fifth (*Fundulus*) injection. These are most unusual circumstances not otherwise encountered in many hundreds of injections.

Table IV—*Continued*

Material injected	Fish number	Days in fresh water		Subsequent recovery completed (days)
		Previous test	With injections	
TSH	21	6	3	Died next day
	18	(*)	4	6
	17	(*)	5	6
	24	6	5	4
Thyroxin	4	7	3	13
	26	5	3	16
	9	6	5	Died 20 days later
	2	4	6	5
GH	44	(*)	4	4
	77	9	4	4
	G3	6	5	5
	68	10	5	4
GH-TSH-ACTH	G13	9	2	Found dead
	G29	9	3	Found dead
	G26	5	3	3
	G30	7	4	Blood sample taken
	46	3	4	Blood sample taken
Posterior lobe preparation	32	(*)	3	4
	14	6	4	5
	78	7	4	4
	G23	7	9	Died next day
0.6% NaCl (Alternate days)	47	4	2	Found dead
	G12	5	3	Found dead
	96	8	2	Died 8 days later
	46	3	5	5
0.6% NaCl (Daily)	11	3	2	3
	9	6	4	6
0.06% NaCl (Daily)	8	4	3	10
	2	4	4	5

cate moderate to strong stimulation of the thyroid and interrenal. Sexual maturation was nearly as great as in normal prespawning *Fundulus*. The experiment was of too short duration to evaluate growth, but length increments in two of the fish indicate the presence of growth hormone, despite the small average weight loss, probably attributable to multiple effects of treatment. The anemia of hypophysectomy was not alleviated.

Injections of pollock brei failed to demonstrate any salutary effect. Nuptial colors, indicating the presence of gonadotropins, appeared in some males during the 2–7 day period before onset of acute symptoms, but since test of subsequent recovery in sea water seemed important, autopsy was not performed to check the presence of other hormones. Due to time limitations the experiment employing the perch pituitary brei was discontinued before the full 20 days, although one fish was still showing none of the symptoms preceding death in fresh water at that time.

The daily pollock brei treatment given to a normal fish in fresh water over a twelve-day period gave evidence of no adverse effects. The saline controls were also unaffected by the injections received. Yet, except for the perch and *Fundulus* breis, none of the injections gave indications of increasing the ability of the hypophysectomized fish to survive in fresh water.

Table V

Condition of fish receiving Fundulus brei at the time of autopsy compared with normal values. (GSI = Gonad Wt. × 100/Body Wt.; HSI = Liver Wt. × 100/Body Wt.; TEH = Average thyroid cell height in microns)

Fish number and sex	Wt. change %	Length change %	GSI	HSI	Anterior interrenal	TEH	Hemoglobin
Fundulus brei:							
4 ♂	− 2.1	−0.8	3.9	3.9	Hypertrophy	5.0	45
55 ♂	−10.1	1.4	6.9	5.1	Hypertrophy	14.4	55
107 ♂	1.5	0	3.1	4.8	Hypertrophy	12.4	50
25 ♀	− 1.3	1.7	4.4	5.2	Hypertrophy	6.0	45
Hypect controls:*							
♂♂	− 1.2	−0.2	0.3	5.4	Inactive but not atrophied	3.8	68
♀♀	−10.4	0	1.1	6.4	Inactive but not atrophied	—	54
Normal wild fish:**							
♂♂ Nov.	—	—	0.7	6.3	—	6.5	—
May	—	—	5.8	2.8	—	6.9	80
♀♀ Nov.	—	—	1.5	6.4	—	6.0	—
May	—	—	6.1	4.8	—	5.9	—

* Males (Pickford, 1954b), means of five fish saline-injected thrice weekly for one month in sea water. Females (Pickford, unpublished) means of three saline-injected daily for one week in sea water.

** Means of about 40 fish per group ♂♂ (Pickford, 1953), ♀♀ (Pickford, unpublished), taken from sea water.

(*h*). In the histological study, the sea water-adapted fish were found to have a gill cytology essentially as described by Copeland (1948): the chloride cells were columnar, acidophilic, reaching from the free surface to the basement membrane, and usually with an "excretory vesicle" at the distal border. In normal fish adapted to fresh water for a week or more, however, most of the cells exhibited shrinkage, rounding up, and frequent loss of contact with the free surface; they were also far less fuchsinophilic. Typical sea water-adapted cells, sometimes with vesicles, were noted, but less commonly. In hypophysectomized fish in fresh or sea water the chloride cells were cytologically the same as normal fish in these respective media.

A cursory count of the mucous cells revealed an average of about four per section in the sea water-adapted and eight in the fresh water-adapted normal fish.

The mucous cells of hypophysectomized fish were emptied and atrophied in contrast to the normal condition. In addition, although the sea water-adapted fish had about four mucous cells per filament section, as did their unoperated counterparts, in hypophysectomized fish in fresh water the number was about two per section, much less than normal. Hypophysectomized fish receiving *Fundulus* brei in fresh water showed the unoperated fresh water-adapted picture; the mucous cells appeared full, well stained, and averaged seven to eight per section.

Discussion

1. Survival in fresh water

There is no doubt that untreated hypophysectomized *Fundulus heteroclitus* cannot withstand transfer to fresh water under the conditions of these experiments. Many fish were saved and used again for unsuccessful injection therapy, injections which apparently have no adverse effects upon hypophysectomized fish in sea water or normal fish in either medium. Adding these 46 instances in which the fish were retested and still failed to survive to the 53 of the first test, a total of 99 cases are available to support this thesis. The more than thirty normal fish, unaffected by transfer from salt to fresh water at various times in company with the experimentals, exclude the possibility of harmful factors in the running fresh water system.

It would seem from the survival time (Fig. 2), varying between two and 10 days with an average of about 6½ days, that the cause of distress is slow in reaching its full effects. The form of the curve, rising slowly and dropping suddenly, supports this contention. It is highly unlikely that the irregularity of the curves derives from any other cause than lack of sufficient numbers to smooth it out.

The wide range of survival times obtained by operating on fresh water-adapted fish appears to show three asthenia peaks (Fig. 3). Out of the group of 25 fish, 44% succumbed during the first three days, 28% survived 6–8 days, and another 28% survived for 11–27 days. These results may be compared with the incidence of asthenia in sea water-adapted fish transferred to fresh water two or more weeks after hypophysectomy (Fig. 2). In this group there was a single peak at 6–7 days, only 10.3% died during the first three days, and none survived longer than 10 days. On the other hand, when sea water-adapted fish are transferred to fresh water immediately after removal of the pituitary they succumb rapidly (Fig. 3, broken line); out of a group of eight such fish none survived more than three days, although it is extremely rare for hypophysectomized fish to die (except later, from collateral effects such as the development of kidney stones, Pickford, 1953) when they are returned to sea water. The survival time in fresh water-adapted *Fundulus* therefore appears to be the result of two opposing factors: an increased sensitivity to fresh water immediately after the operation, and, if this period is withstood, a tendency for the prolongation of survival which could be attributed to pre-existing fresh water adaptation.

The results of Abramowitz (1937) and Matthews (1933), referred to in the introduction, remain unexplained. But the survival time which they noted ("several weeks") is not far outside the limit for fish hypophysectomized in fresh water reported herein. Even with normal *Fundulus* there has been a discrepancy between the results of different authors (Garrey, 1905; Sumner, 1906).

2. *Cause of death*

The low blood chlorides of hypophysectomized fish dying in fresh water (Table IV), less than half of that of operated fish in sea water or normal fish in either medium, is seemingly sufficient cause for death. These studies indicate that *Fundulus* normally has remarkable osmoregularity capacity, no difference being discernible in the blood chlorides of fresh and salt water-adapted fish. Rather than having a slightly higher blood chloride, resulting from decreased osmoregulatory capacity, the hypophysectomized fish in sea water appears to have a somewhat lower blood chloride although the difference is not quite statistically significant ($P = 0.05–0.1$).

Examples of serum chloride determinations on various fish are presented for comparison in Table VI. It is well known that the osmotic pressure of the blood of stenohaline marine teleosts is higher than that of fresh water species, and that euryhaline species are capable of a considerable degree of regulation. The change in blood chlorides is even greater than the fall in osmotic pressure accompanying

TABLE VI

Comparative data on blood chloride for fresh water, sea water and euryhaline teleosts

Author	Species	Serum or plasma NaCl in gm. per cent	
		F.W.	S.W.
Strictly fresh water:			
Vars (1934)	*Cyprinus carpio*	av. 0.477	—
	Esox lucius	av. 0.487	—
Puschel (1928)	*Tinca tinca*	av. 0.517	—
Drilhon (1942)	*Cyprinus carpio*	av. 0.626	—
Stenohaline marine:			
Hall, Grey, and Lepkovsky (1926)	*Brevoortia tyrannus*	—	0.862
Grafflin (1935)	13 species	—	0.875–1.008
Boucher-Firly (1935)	*Conger conger*	—	0.994–1.097
	Muraena helena	—	1.035–1.228
Euryhaline poikilo-osmotic:‡			
Boucher-Firly (1935), Callamand (1943), and Callamand *et al.* (1951)	*Anguilla anguilla*		
	immature	0.375–0.701	0.661–1.170
	hypophysectomized	—	0.631–0.769
	silver, migrating	0.155–0.491*	0.826–1.338
Euryhaline homio-osmotic:			
Koch and Heuts (1942)	*Gasterosteus aculeatus*		
	immature**	0.490–0.650	0.510–0.620
Present paper	*Fundulus heteroclitus*		
	normal	av. 0.887	av. 0.885
	hypophysectomized	av. 0.385†	av. 0.817

* Asthenia is associated with hypochloremia. In a later paper (Fontaine and Callamand, 1948) higher values for some silver eels are given.

** Sexually mature sticklebacks lose their ability to withstand higher salinities.

† Asthenic and on the verge of death.

‡ Osmotic pressure data, summarized by Black (1951) and Kalashnikov (1939), indicate that *Salmo trutta*, *S. salar* and *Acipenser stellatus* belong in this group.

transfer to lower salinities because of the concurrent increase in alkaline reserve (Fontaine and Boucher-Firly, 1934). Two types of euryhalinity are found in fish. In the first, exemplified by the eel, the blood chloride may fluctuate considerably with the environment, whereas in the stickleback and killifish it remains constant. It will be noted that in the hypophysectomized eel, as well as *Fundulus,* the blood chloride of hypophysectomized fish in sea water tends to be lower than normal. The generally lower blood chloride of *Anguilla* in fresh water is undoubtedly related to the fact that this fish is not capable of absorbing chlorides through the gills.

The proportionately small weight changes (Table III) indicate that the salt-regulating mechanism rather than failure of ability to excrete excess water is at fault. In addition, the fish showed no signs of edema such as would result from internal flooding.

The results of the tests in diluted sea water are surprising. The salinities of the $\frac{1}{10}$ and $\frac{1}{4}$ dilutions are below that of *Fundulus* blood. The results show, as might be expected, that survival is merely prolonged. The half and half dilution, equivalent to 1.30 gm. per cent NaCl, is considerably above the salinity of the blood, yet the fish did not survive indefinitely in this medium. The present investigation throws no light on the problem posed by this evidence.

The incidence of renal calculi was not studied. This trouble develops slowly over a period of three weeks or longer and is sporadic (Pickford, 1953). It can be excluded as a cause of death in fresh water since fish operated in fresh water also failed to survive long enough (except in a few instances) for renal lethiasis to develop. One of the four receiving *Fundulus* brei (No. 25) had calculi, seen in sections of the head kidney, yet survived as well as the rest.

3. Hormone therapy

Although there were only four cases, owing to limitations set by the difficulties involved in obtaining a large supply of *Fundulus* glands, it is clear that these injections were responsible for the long survival of the fish in fresh water. The experiment seems to have been adequately controlled to warrant acceptance of this fact: 1) the fish were pretested, 2) they were allowed to stay in fresh water twice as long as the maximum survival time for non-injected sea water-hypophysectomized fish and were still in good health at the end of that time, 3) controls receiving saline under identical conditions showed no increased survivability, and 4) untreated hypophysectomized fish kept in the aquarium at the same time succumbed as usual. It therefore appears safe to conclude that the *Fundulus* pituitary secretes a hormone or hormones enabling the fish to survive in fresh water.

Since all target organs of the adenohypophysis appear to have been stimulated by the *Fundulus* brei, the evidence from the autopsy throws no light on the nature of the particular factor involved.

Since two of the fish receiving perch pituitary brei survived in fresh water considerably longer than the established maximum for hypophysectomized *Fundulus,* it also seems most probable that the factor enabling the fish to live was present in this preparation. The fact that perch brei was not as successful as the *Fundulus* brei could be due to suboptimal amounts of the factor, wrong proportions in the event that more than one hormone is involved, or adverse foreign protein reactions.

Pollock pituitary, whether given under the same conditions or in quadruple amounts (double dosage daily), did not aid survival. This preparation, therefore, either lacks the necessary factor altogether or one of the adverse factors mentioned as possibly present in the perch brei holds an even greater sway in the pollack preparation.

There is every indication that ACTH, TSH, GH, posterior lobe preparation, DOCA, and thyroxin alone or the first three in combination have no salutatory effect. Again it is possible that the dosages were too high or too low, but those of the first four were known to have a moderate influence on target organs. The conditions of injection are even less likely to be at fault. Most were given exactly as the successful *Fundulus* brei. Daily injections may be disturbing since daily weight change tests, not reported here, indicated that such handling was highly undesirable. However, in the presence of the necessary factor for survival it would seem that the hypophysectomized fish could overcome this difficulty, since daily handling does not seriously disturb normal fish in fresh water. Moreover, daily injections would seem essential for thyroxin, which presumably does not act through a target organ. The negative results with all these preparations are surprising in view of the literature which implicates one or the other in teleostean osmoregulation. The results with the posterior lobe preparation serve to substantiate previous findings—that these hormones apparently have no osmoregulatory effect in fish.

All the possibilities have not been exhausted in the tests with these hormones. Dosage levels have not been explored. Three well known vertebrate hormones, prolactin, gonadotropin, and intermedin, remain untested. There is no reason to suspect intermedin, which undoubtedly was present as a contaminant in many of the preparations tested. Gonadotropins also seem unlikely since immature killifish withstand transfer to fresh water as well as mature fish of either sex. In addition, both the pollock and perch breis contained relatively high amounts of the gonad-stimulating hormone and only one of these was successful, whereas the perch brei, evidently with low gonadotropic activity, had a salutatory effect. Prolactin has no known function in fish. Although an osmoregulatory control would not be suspected of the hormone from its known activity in higher vertebrates, it is not ruled out. More likely, however, since the pituitary breis of *Fundulus* and the strictly fresh water perch possess the factor, whereas it is probably absent in the marine pollock, an as yet undescribed hormone is involved.

4. Gill cytology

The cytological picture of the gills of normal *Fundulus* adapted to sea water was found to be exactly as that described by Copeland (1948) and requires no further comment. The results for fresh water adaptation obtained in this study more closely resemble the condition described by Copeland for fish in distilled water than his description of the condition in tap water. The discrepancy could be due to differences in the calcium content of the water or a similar cause which could be brought out only by complete tap water analyses. There is no doubt that the chloride cells are shrunken and less strongly fuchsinophilic in fresh water. When gills of fish adapted to each of the two media were stained on the same slide by the Altmann method, the chloride cells of the fresh water specimen were scarcely to be seen, while the sea water sections were excellent.

The fresh water cytological picture noted in this study is not unlike that described by Keys and Wilmer (1932), who found the chloride-secreting cell-type less abundant or absent in fresh water fish as compared with marine forms, while some acidophils were also seen rounded up below the epithelium in the former. The findings herein are also reminiscent of the hardly distinguishable cell in *Macropodus* before saline adaptation (Liu, 1942), the merely lessened abundance of chloride cells with vesicle noted by Getman (1950) in the eel, and the lesser chloride cell development noted by Nishida (1953) in the land-locked as compared with the sea-run salmon.

The fact that the chloride cells appear normal in hypophysectomized sea water-adapted fish is in keeping with the chloride excretion function, long attributed to these cells, since blood-chloride studies indicate that the fish maintain osmotic equilibrium perfectly well in this hypertonic environment. The obvious change in these cells after transfer to fresh water in normal fish was again found in hypophysectomized ones, indicating normal response as far as cutting down of chloride excretion is concerned.

Mucous cells are apparently holocrine. These are the only cells in the mammalian gastric mucosa which have a significant mitotic rate, presumably to replace cells extruded into the cavity of the gut (Stevens and Leblond, 1953). However, observations on the digestive system of hypophysectomized rats (Baker and Abrams, 1955) showed that the mucous cells are not affected. Nevertheless it appears that those in the gills of hypophysectomized *Fundulus* have discharged their contents and have not been replaced. Mucous cells may play a special role in fresh water fish. They were noted to be more predominant in the gills of fresh than in sea water-adapted fish in this investigation, and Keys and Wilmer found them so in fresh water over marine teleosts. The skin was not studied in the present investigation, but it appears likely that all epidermal mucus secretion would be under pituitary control if such is the case in the gills.

The protective role of mucus in the osmoregulation of fishes is well known. Firly (1932) found that removal of mucus from the skin by washing was more harmful to eels transferred from fresh to salt water than *vice versa*. The opposite effect was observed in this study; however, isotonic and hypotonic media were found to be deleterious, whereas the fish apparently maintain themselves well in hypertonic sea water. It appears obvious from the weight change and blood chloride studies that hypophysectomized *F. heteroclitus* in fresh water are able to cope with the increased water load imposed by the medium but suffer from an inability to maintain the normal blood chloride level. Since the site of chloride loss, whether through the gills, skin, or kidney is unknown, it is not possible to state definitely that atrophy of the mucous cells is involved. An increased permeability of the skin, resulting from lack of a protective coating, would lead to polyuria (Grafflin, 1931) with possible abnormal loss of chloride. But the failure to survive, even in an approximately isotonic medium, indicates that other factors are operative.

SUMMARY

1. Hypophysectomy results in a loss of ability to survive in fresh water by the euryhaline cyprinodont, *Fundulus heteroclitus*. The average survival time is 6–7

days for salt water-adapted fish. Preadaptation in fresh water before operation may prolong survival but does not prevent ultimate death.

2. Hypophysectomized fish are unable to survive in salinities up to 13 ‰.

3. Symptoms of death are those of asthenia accompanied by a slight weight increase, averaging 4.3% in fish not being handled daily.

4. Serum chloride determinations showed that normal fish in either fresh or salt water maintain a uniform level of *ca.* 0.886 gm.% NaCl. Serum of hypophysectomized fish in salt water is not significantly lower (*ca.* 0.817 gm.%). Hypophysectomized fish dying in fresh water have only *ca.* 0.383 gm.%.

5. Replacement therapy with *Fundulus* pituitary brei enabled hypophysectomized fish to survive in fresh water. Pituitary extract from *Perca flavescens,* a strictly fresh water species, was partially effective, whereas extracts from glands of *Pollachius virens,* a marine species, had no beneficial influence.

6. Injections of ACTH, GH, TSH, thyroxin, DOCA, posterior lobe extract, and an ACTH-GH-TSH combination failed to enable the fish to survive in fresh water.

7. The data presented above appear to indicate that the pituitary of *F. heteroclitus* secretes an unknown factor(s) which regulates salt balance in that fish in fresh water. This factor is apparently lacking in the stenohaline marine species, *P. virens,* but may be present in the fresh water species, *P. flavescens.*

8. Hypophysectomy has no effect on the cytology of the chloride cells either in the active (sea water) or regressed (fresh water) condition.

9. Mucous cells were more abundant in the gills of normal fish when adapted to fresh water than when adapted to salt water. Hypophysectomy resulted in atrophy of the mucous cells in either medium and a decreased abundance in fish dying in fresh water. The abundance and condition of the mucous cells was restored to normal in hypophysectomized fish which were induced to survive in fresh water by injections of *Fundulus* pituitary brei.

10. The data suggest that the chloride cells are not concerned in the failure of hypophysectomized fish to survive in fresh water, but that atrophy of the mucous cells may be involved.

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CYTO-EMBRYOLOGICAL STUDIES OF SEA URCHINS.
III. ROLE OF THE SECONDARY MESENCHYME CELLS IN THE FORMATION OF THE PRIMITIVE GUT IN SEA URCHIN LARVAE

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Analyses of the mechanism of gastrulation in the past have been based either on observations of the normal process or on studies of the effect of LiCl. When one proceeds along the first line, it is rather difficult to reach a decisive conclusion. When one selects the second approach, in addition to the failure of gastrulation, another, more profound, factor of changes in differentiating potency is introduced. Yet, since lithium treatment and exogastrulation are so consistently associated, there is a tendency to equate lithium treatment with the analysis of the mechanism of gastrulation. Obviously handling two variables simultaneously is a rather dangerous procedure. In a strict sense, therefore, before attacking the lithium effect, exogastrulation not accompanied by a change in differentiation capacity should be studied. This attitude is observed in the present paper.

NORMAL PROCESS OF GASTRULATION

Concerning the mode of gastrulation in sea urchins, the views of such earlier workers as Herbst (1893, 1896), Morgan (1896), Schmidt (1904) can roughly be summarized as saying that the endodermal plate of the beginning gastrula gives rise only to the tip of the archenteron, while the blastular wall encircling the original endodermal plate invaginates, completing the formation of the gut. These investigators believed that the volume of the larva actually decreases during gastrulation because of the inward migration of so much of the blastular wall.

On the other hand, Hörstadius (1935, 1936, 1939) showed by a vital staining technique that the presumptive archenteron takes its origin from the veg$_2$-disk of the 64-cell stage. At the beginning of gastrulation, this entire mass invaginates and the gut is formed by the elongation of the mass. Hörstadius further asserted, in contrast to the conclusion of the previous workers, that the volume of the larva increases during gastrulation.

Although the process of gastrulation can be observed fairly clearly in the living larvae of any transparent forms, the observation can be further supplemented by following the development of individual larvae reared in a simple moist chamber as described below.

From a piece of tissue paper, the thickness of which is a little less than that of the larva, a square piece slightly smaller than a cover slip is cut out and a hole

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several millimeters in diameter is made at the center. After wetting the paper with sea water on a slide, a droplet of sea water containing one larva is placed at the center of the hole (but not touching the paper) and a cover glass is slowly laid down. The wet paper prevents evaporation and the droplet is the culture medium for the larva. If the entire preparation is put into a still larger moist chamber, or if distilled water is occasionally added to the paper (these methods are preferable to sealing the cover slip with vaseline), it is possible to rear the larva from the gastrula to the pluteus stage. In this small space, as the larva swims rather slowly, photographs of various views of the larva can be made with a shutter speed of $1/50$ second. Further, in such droplets, although development is slowed down to some extent, no sign of morphological abnormalities was detected.

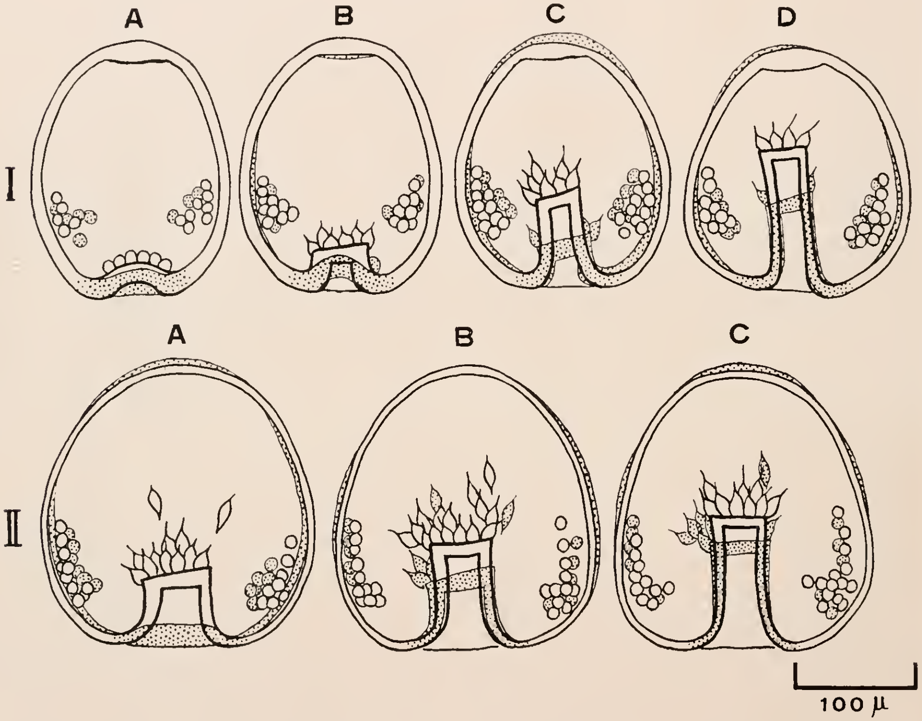

FIGURE 1. Superimposed contour-tracings of photographs of a single larva through the gastrulation process. Dotted drawings represent preceding stages and open drawings are succeeding stages. The upper row: *Clypeaster japonicus;* the lower row: *Mespilia globulus.*

Figure 1 shows superimposed tracings of a series of photographs, taken at appropriate intervals, of the larvae of two kinds of sea urchin. As can be seen from the figure, throughout the period from the stage of endodermal plate formation to the completion of gastrulation, the contours of the larvae and the positions of the primary mesenchyme cells hardly change, while the archenterons increase their lengths considerably. Moreover, it will be noticed that as the archenteron elongates, the thickness of its wall decreases. The authors believe that this fact supports the conclusion of Hörstadius mentioned. The only discrepancy found is that the volume of the larva remains constant during the gastrulation process, and it is only after the completion of gastrulation that the volume increase takes place.

From both Hörstadius' results and these observations of the authors, the following facts seem to be certain:

1) All the material which will form the archenteron is first laid down as an endodermal plate preceding gastrulation.

2) At the beginning of gastrulation, the entire mass of this endodermal plate invaginates *in toto*.

3) The formation of the primitive gut is due to the stretching of the original endodermal plate, with no other elements included.

Formation of Pseudopodia by the Secondary Mesenchyme Cells

Soon after the onset of invagination of the endodermal plate, the secondary mesenchyme cells, differentiating at the tip of the archenteron, begin to send out fine pseudopodia.[3] These pseudopodia elongate toward the wall of the animal side of the larva, and by the time the archenteron attains a length ½–⅓ the diameter of the blastocoel, the ends of the pseudopodia are attached to the blastular wall either directly or by the mediation of a few cells arranged on top of the others.

After the establishment of the connection between the blastular wall and the pseudopodia, larvae are occasionally found in which the part of the blastular wall where the pseudopodia are attached is pulled in. In *Pseudocentrotus* larvae this effect is very conspicuous. In mid- or late gastrula stage, they frequently acquire two indentations, one on the animal side where the pseudopodia are anchored, and the other, the blastopore, on the vegetal side; thus the entire larvae somewhat simulate the shape of a mammalian red blood cell (see also Okazaki).

Moreover, if the shape of the tip of the archenteron is carefully observed, it will be seen that it seldom has a rounded contour, but it is of an edgy or squarish shape resembling a suspended fabric. In case a group of pseudopodia develops better on one side, the roof of the archenteron is lifted up higher toward the animal pole on that side. All these facts give the impression that the archenteric roof is being pulled toward the animal pole by the pseudopodia of the secondary mesenchyme cells. This interpretation is further consolidated by the following several experiments.

Injection of Oil Droplets into the Blastocoel

In order to find out whether mesenchymal pseudopodia are actually capable of pulling an object, the simplest way will be to introduce an easily movable object into the blastocoel. For this purpose, oil droplets were micro-injected into the blastocoels of newly hatched blastulae. When the primary mesenchyme cells appear, regardless of the position in the blastocoel of the oil droplets, they are caught by the primary mesenchyme cells. However, when the secondary mesenchyme cells are formed, their traction force is apparently stronger than that of the primary

[3] The time of appearance of the pseudopodia differs from one species to another. In *Mespilia,* in which they appear earliest, the pseudopodia are formed immediately after the initiation of invagination; in *Pseudocentrotus,* in which they appear late, the pseudopodia can be recognized clearly only after the archenteron reaches ⅓ of the distance across the blastocoel.

mesenchyme cells so that by the time the mid- or late gastrula stage is reached, the oil droplets are relayed over to the secondary mesenchyme cells and are drawn toward the archenteric tip. Some photographs are shown in Figure 2. It should be pointed out that since the injected oil drop is immediately surrounded by a protein film, the pseudopodia have no difficulty in getting hold of it.

Now having seen that the basic conditions are fulfilled, let us proceed to examine whether or not the pseudopodia are actually pulling up the archenteron.

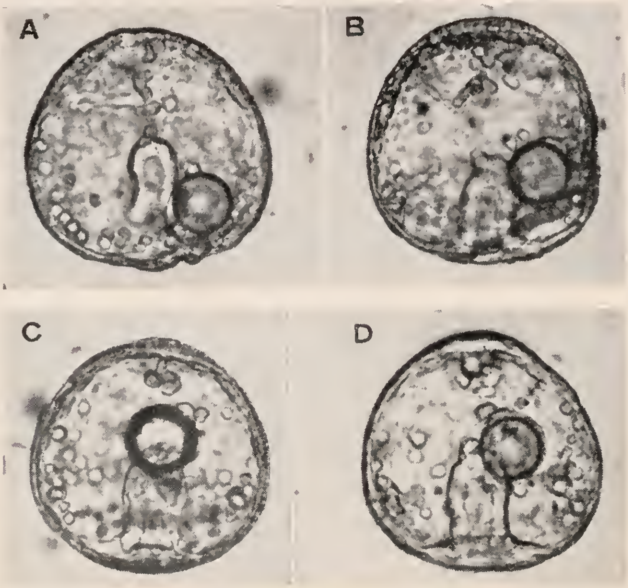

FIGURE 2. Microphotographs of *Clypeaster* larvae with oil drops injected into the blastocoels. A. Early stage. Oil droplet is caught by the pseudopodia of the primary mesenchyme cells. B. Intermediate stage. The oil is picked up by the pseudopodia of the secondary mesenchyme cells also. C, D. Later stages. As the pull by the secondary mesenchyme cells is stronger, the oil is removed from the primary mesenchyme cells and is drawn toward the archenteric tip.

EFFECTS OF EXPERIMENTAL INCREASE OF THE BLASTOCOELIC VOLUME

If the tip of the archenteron is actually being pulled up toward the animal pole by pseudopodia which have anchored themselves on the blastular wall, the pulling force will be increased if the blastocoelic volume is increased. This increased pull, in turn, would stretch the archenteron making it longer. Moore (1939, 1940) and Dan (1952) have reported that since the sea urchin blastular wall loses permeability to sucrose molecules after the tenth cleavage cycle (*i.e.*, at the time of hatching), if larvae cultured in a sucrose-containing medium are transferred to pure sea water after hatching, they become considerably inflated as the result of an abnormally high internal osmotic pressure developed by the sucrose molecules entrapped in the blastocoel.

Repetition of the sugar procedure on *Clypeaster japonicus* and *Mespilia globulus* gives not only a confirmatory result concerning the above expectation but it further gives rise to exogastrulae in the proportion given in Table I. Figure 3 illustrates superimposed tracings of photographs of individual larvae of *Mespilia* during the process of inflation and of exogastrulation. As can be seen in the figure, during the few minutes following the transference from sucrose sea water to pure sea water when the larva is increasing in size, the pseudopodia are stretched and at the same time, the archenteron actually lengthens (Fig. 3, B, C). However, when the volume is further increased, the pseudopodia or chains of mesenchyme cells extending between the gastrular wall and the tip of the archenteron are broken and the cells round up. Under this circumstance, the archenteron begins to be pushed out of the blastocoel from its basal part (Fig. 3, F and Fig. 4).

Whenever the archenteric tip is wide and flat-topped, it was invariably found that some of the pseudopodia are anchored to the lateral wall instead of to the animal pole region so that they are pulling the archenteric tip sideways (Fig. 3, D).

TABLE I

Percentages of normal, entoexo- and exogastrulae obtained by inflation of the blastocoel by sugar treatment in Clypeaster japonicus and Mespilia globulus

Species	Stage of returning from sugar solution to sea water	10% sugar s.w. → Natural s.w.			20% sugar s.w. → Natural s.w.			30% sugar s.w. → Natural s.w.		
		Invaginated form	Ento-exo-gastrula	Exo-gastrula	Invaginated form	Ento-exo-gastrula	Exo-gastrula	Invaginated form	Ento-exo-gastrula	Exo-gastrula
Clypeaster	Early gastrula	—	—	—	72.5%	15%	12.5%	92%	5.5%	2.5%
	Mid gastrula	—	—	—	65	14.5	20.5	25	21	54
Mespilia	Early gastrula	67%	5%	28%	43	12	45	—	—	—
	Mid gastrula	68	10	22	30	12	58	—	—	—
	Early gastrula	74	8	18	65	8	27	—	—	—
	Mid gastrula	28	32	40	18	24	58	—	—	—
	Late gastrula	69	26	5	62	25	13	—	—	—

At any rate, as long as the pseudopodial connection is intact, the archenteric tip takes an edgy contour like a suspended fabric, while if the connection is severed, the contour rounds off (Fig. 3, E, F).

Generally speaking, secondary mesenchyme cells are amoeboid. Therefore, they are continuously extending and retracting pseudopodia, forming new ones and abolishing old ones, and some cells move along other cells. When such pseudopodia are broken as the result of blastocoelic expansion, although the secondary mesenchyme cells round up for a while, sooner or later they begin to send out new pseudopodia. In a larva in which only the basal part of the archenteron is evaginating, if a new pseudopodial connection is established in the normal position, the archenteric tip begins to be pulled back toward the animal pole once more. As a matter of fact, even in larvae in which the entire archenteron has been everted, the archenteric tip can still resume the inward movement when a connection is successfully established. Because of these secondary connections, various degrees of entoexogastrulae result in an experimental batch. Some of these are depicted in Figure 4, A–F.

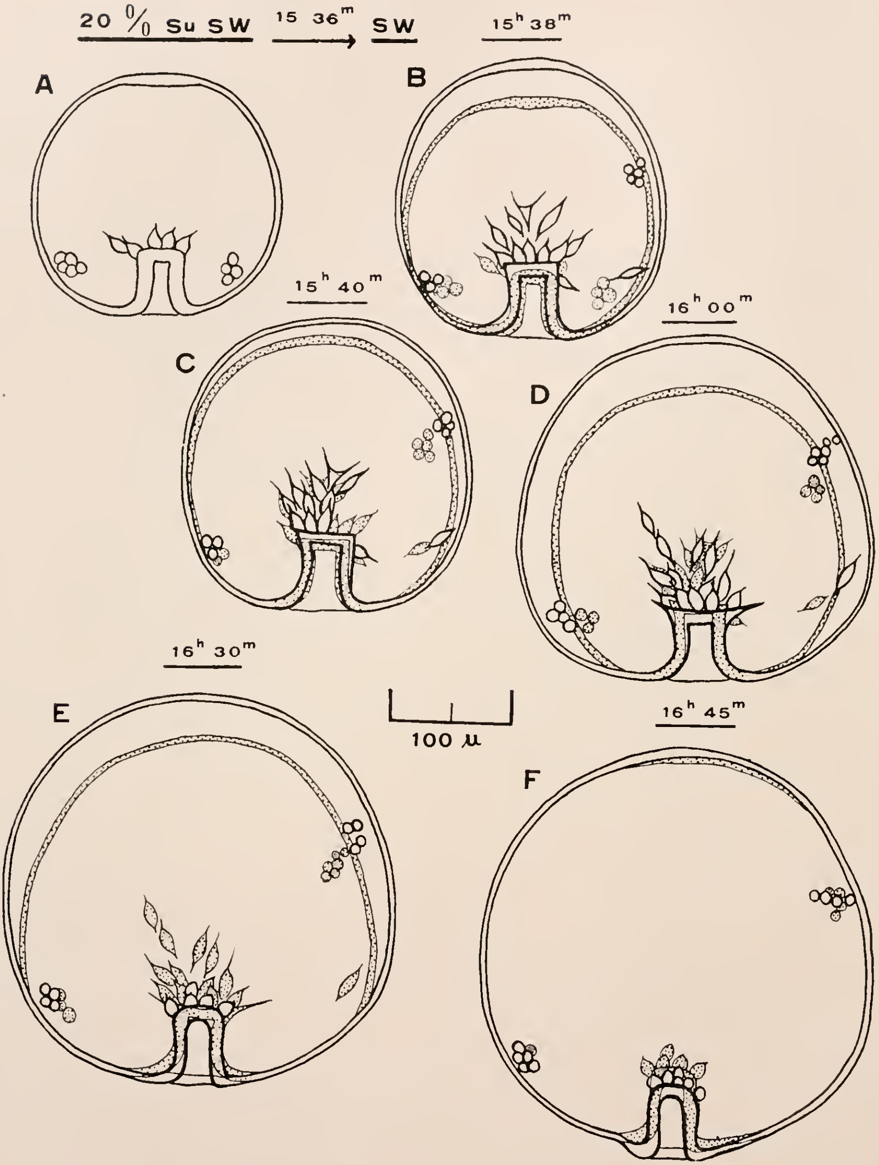

FIGURE 3. Effects of inflation of the blastocoel on the pseudopodia of the secondary mesenchyme cells and on the archenteron of *Mespilia globulus*. A. A larva in a sugar-containing sea water before transfer. B, C. Transfer to pure sea water. As a result of blastocoelic expansion, the pseudopodia are stretched, in turn pulling the archenteron longer. D. Flat-topped archenteron indicates that the pseudopodial connection to the animal pole has been severed while the connection to the side wall is still preserved. E. Rounded tip of the archenteron indicates the severance of the pseudopodial connection and the archenteron is receding. F. The secondary mesenchyme cells have also rounded up and the gut is evaginating.

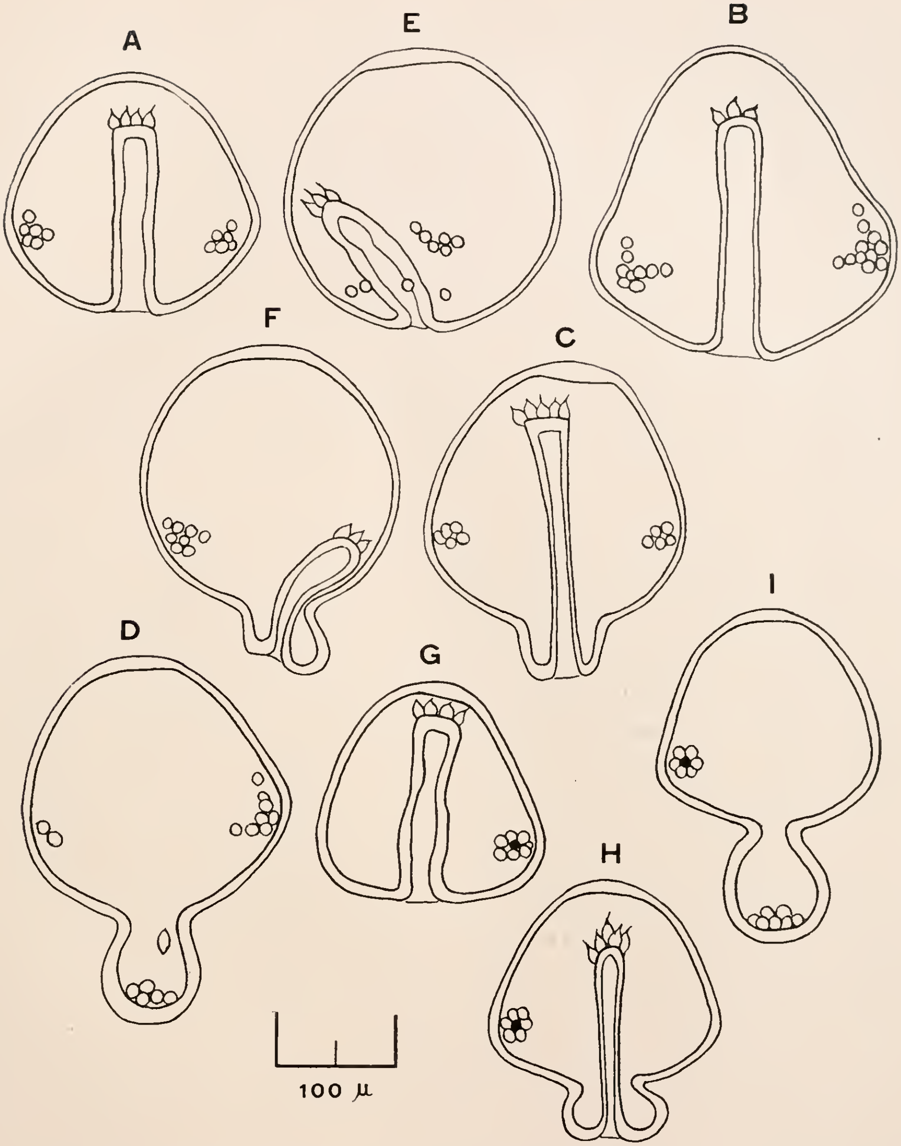

FIGURE 4. Various types of abnormality in *Clypeaster japonicus*. A-F: Transferred from sugar to pure sea water at early gastrula stage. A. Control in sugar-sea water. B. Inflated larva which has successfully invaginated. Note the height of the archenteron and the thinness of its wall. C. An entoexogastrula. The wall of the gut is thinned out considerably. D. An inflated larva with a completely evaginated gut. The wall of the gut is very thick. E. An inflated larva in which the pseudopodia have attached themselves to the side wall. F. The same as E with the gut going astray. G-I: Exposed to Ca-low condition (1/10 that of sea water) from the gastrula stage onward. G. Control for Ca-low series. H. An entoexogastrula caused by the Ca-low treatment. I. Exogastrula in 1/10 Ca.

From Figure 4 it becomes evident that the completely everted archenteron of an exogastrula (D) is shorter and its wall is thicker than the control (A), while the invaginated part of the gut of an entoexogastrula is highly stretched and its wall is extremely thin (C). This result is to be expected in the latter type, since a part of the gut having been everted, the remaining fraction has to supply all the material to be pulled out to reach the animal pole. Comparison of Figures 4 A and 4 B shows that even among successfully invaginated larvae, a proportionality is evident between the size of the blastocoel and the length of the gut or the thinness of the archenteric wall.

From the above observations, the following points can be inferred. When the blastocoel is abruptly inflated by a transfer from a sucrose medium to sea water, the pseudopodia of the mesenchyme cells tend to break and the primitive gut retreats. But judging from cases of entoexogastrulae, if the pseudopodial connection is re-established, its mild but continued pull can eventually pull out the primitive gut so that it becomes longer than normal (C). The short, bag-like shape of the completely everted archenteron of an exogastrula (D) must be produced under the complete absence of such an extrinsic traction. By the time the normal larva reaches the pyramid stage, however, the everted gut of the exogastrula grows in length to a limited extent and eventually exhibits a perfect tripartite formation. This fact indicates that even in the everted gut, tissue differentiation can take place perfectly, although such a gut never assumes the cylindrical shape typical of normal gastrulae of an advanced stage. This, in turn, indicates that the typical cylindrical shape and squarish tip of the normal archenteron is brought about by the pulling force of the pseudopodia. On the other hand, an abnormally stretched archenteron of an entoexogastrula often breaks in the thinnest part, and the upper portion goes to the animal pole while the basal portion evaginates completely. The result is a larva which has a stomodaeum and a pharynx at the normal position but with the stomach and intestine everted. This is obviously a case in which the primitive gut has yielded to the pulling force.

TABLE II

Percentages of normal, entoexo- and exogastrulae caused by Ca-low treatment in Clypeaster

S.W.........................1/10 Ca

Stage of transfer	Experiment 1			Experiment 2		
	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula
Early gastrula	31%	16%	53%	18%	0%	82%
Mid gastrula	39	34	27	74	7	19
Late gastrula	87	10	3	96	2	2
Post gastrula	97	3	0	100	0	0

Among larvae which have been subjected to the sucrose treatment, such forms as those shown in Figure 4 E and 4 F are also encountered, in which the tip of the archenteron has failed to reach the stomodaeum although the gut as a whole remained inside the blastocoel. The cause for this lies in the fact that when new pseudopodia were formed, they attached themselves to the side wall instead of to the wall of the animal pole and as a consequence, the archenteric tip is led to a wrong destination.

Suppression of Pseudopodial Formation of the Mesenchyme Cells

From what has been said, it can be expected that exogastrulae will also result if the pseudopodia are made to retract instead of being broken. In order to cause already formed pseudopodia to disappear, there are three methods available.

1) In *Clypeaster,* if the larvae are transferred to Ca-low sea water ($\frac{1}{10}$ Ca concentration of sea water) at the gastrula stage when pseudopodia are being formed by the secondary mesenchyme cells, the pseudopodia disappear in some of the larvae and exo- and entoexogastrulae similar to those obtained by the inflation technique are produced (Fig. 4, G–I). The frequency of their occurrence is given in Table II. Closer tracing of the development of a single larva reveals that the suppressing effect of the Ca-low medium works rather slowly and for a few hours pseudopodia can be seen, during which time the archenteron keeps on elongating little by little. But as soon as the pseudopodia disappear completely, the archenteron begins to retreat. The correlation between the two is quite definite, as will be seen in Figure 5, I.

In the Ca-low batch, various gradations of suppression are seen: complete suppression giving exogastrulae (Fig. 5, I), resumption of pseudopodia formation resulting in entoexogastrulae (II), formation of very slender and weak pseudopodia enough to prevent exogastrulation but failing to bring the archenteric tip to the stomodaeum (III), and finally quasi-normal larvae (Fig. 5, IV). In the III class of larvae, the secondary mesenchyme cells form an unpaired coelom at the tip of the primitive gut, thus preventing the union between the archenteron and the stomodaeum.

2) In *Mespilia,* if the gastrulae are exposed to pancreatin (0.5 to 0.125% in sea water), the pseudopodia are abolished in a short time and exogastrulae are formed (Fig. 6). The percentages of entoexo- and exogastrulae are given in Table III. Besides the suppression of the pseudopodia, the pancreatin has secondary effects such as decreasing the larval volume and causing the blastular wall to wrinkle. This latter effect is presumably due to the action of pancreatin on the intercellular matrix and on the hyaline layer. However, concerning the hyaline layer, Moore (1952) has shown that this layer does not play an essential role in the gastrulation process. Because of these secondary effects, it is impossible to rear the larvae in the pancreatin solution indefinitely. However, if they are returned to sea water in time, larvae similar to those shown in Figures 4 and 5 for *Clypeaster* can develop.

3) Among *Mespilia* larvae kept in normal sea water, it sometimes happens that gastrulation proceeds seemingly normally during the early phase, but later spontaneously deviates from the normal course and leads to exo- or entoexogastrula formation. Even in such spontaneous cases, coincidence in the time of pseudopodial disappearance and evagination is always recognized.

Total Inhibition of Pseudopodia Formation

This can be achieved in *Pseudocentrotus* larvae by rearing them in a Ca-low medium ($\frac{1}{20}$ to $\frac{1}{10}$ normal Ca level) from an early stage. In this case, no sign

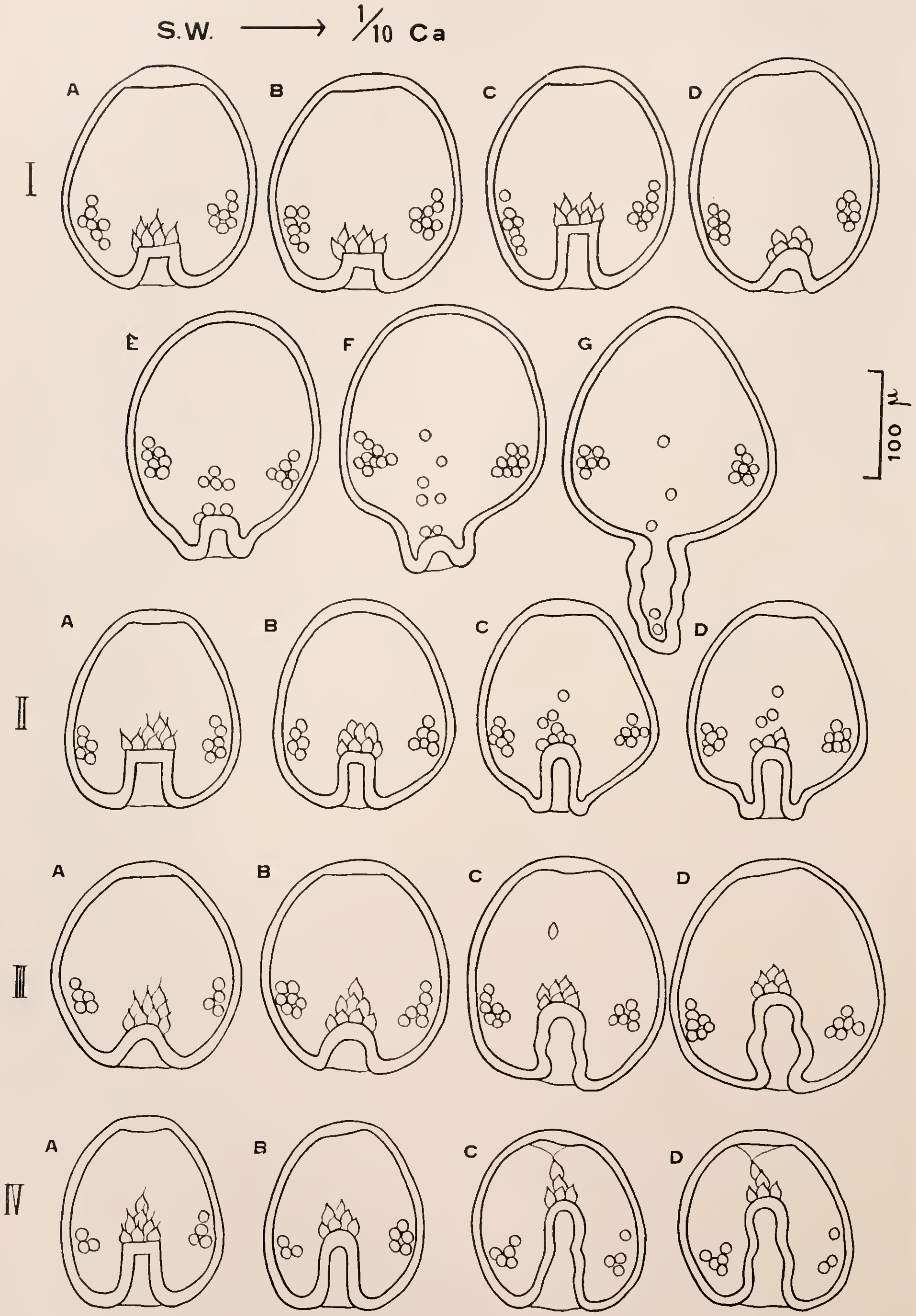
S.W. → 1/10 Ca
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FIGURE 5.

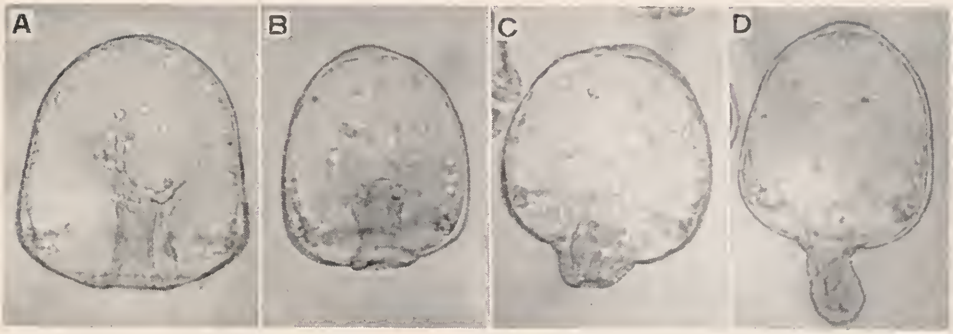

Figure 6. Course of exogastrula formation by pancreatin-sea water in *Mespilia globulus.*

of pseudopodia can be found and the archenteron extends ⅓ to ½ of the way across the blastocoel and stops there. By the time normal larvae in sea water have completed gastrulation, the archenterons of the larvae in the Ca-low medium gradually begin to retreat and they finally form exogastrulae. (A more detailed account of this process will be given in another paper by Okazaki.)

Table III

Percentages of normal, entoexo- and exogastrulae caused by pancreatin treatment in Mespilia

Stage of transfer	Pancreatin concentration								
	0.5%			0.25%			0.125%		
	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula	Invaginated	Entoexogastrula	Exogastrula
Early gastrula	3%	15%	82%	12%	10%	78%	37%	7%	56%
Mid gastrula	12	18	70	24	26	50	34	6	60
Late gastrula	40	14	46	13	19	68	55	10	35
Post gastrula	88	7	5	91	5	4	89	2	9

Discussion

The experimental results reported in this paper have shown that the pseudopodia of the secondary mesenchyme cells play an indispensable role in the formation of the primitive gut of sea urchin larvae. However, it is admitted that the pulling of the mesenchymal pseudopodia alone is not sufficient to account for all the power required to make a young endodermal plate change into the cylindrical gut of a late gastrula. For one thing, the endodermal plate begins to invaginate at the very be-

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Figure 5. Suppression of the pseudopodia of the secondary mesenchyme cells of *Clypeaster japonicus* by ¹⁄₁₀ Ca that of sea water. The first figure (A) for each series is a larva immediately before transfer to the Ca-low medium. I. Complete suppression and resulting exogastrula formation. II. Partial recovery of the pseudopodia leading to an entoexogastrula. III. Feeble pseudopodia just strong enough to keep the archenteron from eversion but failing to draw it to the stomodaeal position. Such a larva acquires an unpaired coelom intervening between the stomodaeum and the archenteron. IV. A seemingly perfect larva in ¹⁄₁₀ Ca.

ginning of gastrulation, before the secondary mesenchyme cells start to form pseudopodia. Consequently, the pseudopodia cannot in any way determine whether the endodermal plate invaginates or evaginates. For another thing, such an endodermal plate can elongate by its own power without the cooperation of the pseudopodia. Probably it is justifiable to think that the maximal limit of this autonomous elongation is shown by the length of completely everted gut of an exogastrula.

Then what is the factor which causes this autonomous elongation of the endodermal plate? As has been realized by many previous workers, the only clue available at present is the change in shape of the cells composing the endodermal plate. While they are in the endodermal plate, they are tall and cylindrical. But as the invagination proceeds, they flatten out laterally. This change takes place even when the endodermal plate evaginates from the beginning. However, a point of importance is the fact that this flattening normally occurs in such a direction that it can be magnified by the pull by the pseudopodia.

Thus, the authors view the situation as follows: within the process of gut formation, three factors must be operating; (1) a factor responsible for the initiation of the invagination of the endodermal plate, (2) a process bringing about the form change of the constituent cells of the endodermal plate and (3) the traction exerted by the pseudopodia. During the early gastrula stage, the first two are acting while in the late gastrula stage, the second and third must be cooperating.

Concerning the first factor, which initiates invagination in the endodermal plate, various views have been expressed. Long ago Rhumbler (1902) attempted an analysis of the process on the premise that three factors—(a) pressure within the blastular wall resulting from increase in cell number, (b) pressure by a confining membrane and (c) absorption of blastocoelic fluid—might be playing some role. But after showing that none of these was involved, he tried to attribute the cause of inpocketing to surface tension changes in the vegetal cells. Morgan (1927) and a few others considered that it might be due to the pressure of dividing cells acting in a plane passing through the apical and basal poles of the embryo. Agrell's recently published opinion (1954) hints at a similar possibility. However, Morgan's idea was negated by Moore (1939). Moore himself contends that the invaginating force resides within the endodermal plate itself and this force decreases along the radial gradient with a maximum at the center. He attempted to calculate this force, using the results of his sugar experiments (1941).

Since then, Lewis (1947) has suggested for amphibian invagination that the superficial gel layer might be playing a role. Moore (1952) went on to test the validity of Lewis' idea in sea urchins and concluded that the hyaline layer of sea urchins is not essential for gastrulation (see also Dan, 1952). Gustafson and Lenique (1952) try to explain it by an increased pressure within the wall caused by a structural protein which is derived from mitochondria.

With respect to the form change of the cells making up the endodermal plate, Gustafson suggests the same explanation, that is, stretch by a structural protein. However, it must not be forgotten that besides simply elongating, such a gut is also able to self-differentiate.

Finally, the process of development of a functional digestive tract in sea urchin larvae requires a fusion between the tip of the archenteron and the gastrular wall at the stomodeal position. The authors believe that the observations presented in this paper show conclusively that this is accomplished by the directing and pulling activity of the secondary mesenchyme cells, before they begin their main task of forming the coelom.

However, the authors do not hesitate to admit that the elucidation of the mechanism of the gastrulation process is still remote from a solution. What causes the formation of the endodermal plate to begin with? What factor initiates its invagination? What factors are involved in the autonomous elongation and self-differentiation of the primitive gut? How do the secondary mesenchyme cells know the site of the stomodaeum? All these important questions are left unanswered.

SUMMARY

1. The first half of the gastrulation process in sea urchins begins with an *in toto* invagination of the endodermal plate, followed by its stretching.

2. In the second half of the process, pseudopodia are sent out by the secondary mesenchyme cells toward the animal pole. These pseudopodia attach to the blastular wall and pull the archenteron up toward that pole.

3. The pulling capacity of the pseudopodia is shown by the displacement toward the secondary mesenchyme cells of oil droplets injected in the blastocoel.

4. When the pseudopodial connection is artificially severed by one of the following methods, exogastrulae result:

a) Blastocoelic expansion after sucrose treatment (*Mespilia globulus, Clypeaster japonicus*).

b) Ca-low treatment (*Clypeaster, Pseudocentrotus depressus*).

c) Pancreatin treatment (*Mespilia*).

5. Abnormal invagination in various degrees brought about by the above treatments can be accounted for by differences in their effects on the pseudopodia formation.

6. Pseudopodia of the secondary mesenchyme cells are indispensable in the latter half of the gastrulation process in order to produce normal embryos.

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GROWTH RATES IN LATITUDINALLY AND VERTICALLY SEPARATED POPULATIONS OF MYTILUS CALIFORNIANUS[1]

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Many marine poikilothermal species which encounter a relatively wide temperature difference over a latitudinal or vertical distribution, or over the annual cycle of seasons, demonstrate a compensatory response in various physiological activities. Rao (1953) has shown that the pumping rate of *Mytilus californianus* was higher for northern latitude animals at any given temperature when this activity was compared with southern ones. Segal (1955) compared heart rate of high and low intertidal populations of *Acmaea limatula* and found that low intertidal animals behaved as if they were cold-adapted relative to high intertidal animals. This has also been shown for *Mytilus californianus* for rate of water propulsion (Segal, Rao and James, 1953). These studies suggest a homeostatic tendency in many rate functions. The literature relevant to the problem of acclimation is extensive and has been reviewed recently (see Bullock, 1955 and Prosser, 1955).

With regard to rate of growth, usually, it is assumed that marine poikilotherms which inhabit high latitude seas grow more slowly, grow to a larger size and have a greater longevity, when compared with individuals of the same or closely related species from warmer seas. Such an assumption suggests that rate of growth is a simple function of temperature. Most studies reported in the literature indicate temperature-dependence, and do not, as in the case in metabolic studies, suggest compensation for temperature difference. However, Dehnel (1955) has compared rates of growth of larvae of several species of intertidal gastropods from southeastern Alaska and southern California. These results have shown that northern larvae grew more rapidly at all experimental temperatures, when compared with southern larvae of the same species. A discussion of some of the literature concerning rates of growth and latitude will be found elsewhere (Dehnel, 1955).

The purpose of this study is two-fold: to determine whether rates of growth in populations of the California sea mussel, *Mytilus californianus,* from southeastern Alaska and southern California are adapted in a compensatory direction relative to the temperature difference; to determine the effect of tidal height upon growth rate in this species. Few studies have concerned the latter aspect of any species. Coulthard (1929) compared growth in vertically separated populations of *Mytilus edulis.* These results showed rates of growth to be greater in completely submerged populations. Moore (1934) compared growth in populations of *Balanus*

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balanoides located at different tidal levels, and found that growth was greatest at higher tidal levels except during the first year. Hatton (1938) studied rates of growth in high and low intertidal populations of *Balanus balanoides, Chthamalus stellatus* and *Patella vulgata.* This work revealed that young barnacles located at lower levels grew faster, but older animals grew more rapidly at higher intertidal levels. Reciprocal transplants of high and low intertidal animals showed that lower levels were still unfavorable for growth. Results obtained for *Patella vulgata* indicated that low level populations of this species grew more rapidly when compared with high level ones.

Rates of growth in adults of *Mytilus californianus* were studied by marking and measuring individuals *in situ* in the intertidal and allowing them to remain in the beds, in this manner obtaining natural rates of growth. The period of the study was chosen to be only a limited part of a year, during the growing season.

Material and Methods

Two geographic localities were chosen for this study: Big Rock, Los Angeles County, California (latitude 34° 00′ N., longitude 118° 30′ W.), and Mount Edgecumbe, Alaska (latitude 57° 03′ N., longitude 135° 20′ W.). Time spent at the northern locality extended from June to September, 1952, and July to August, 1953. The mean difference in surface sea water temperature between the two localities during the summer months was about 7° C.

It was regarded as important that rates of size increase in this species be compared at a season when growth was the most rapid. Coe and Fox (1942) showed that mussels from La Jolla, California grew most rapidly during April and May. The temperature of the sea water at that time was about 16° C. Water temperatures around Big Rock, California were not significantly different. In addition to the spring months thus selected for this study, the southern California mussels were measured in November, following the return from the northern locality. In southeastern Alaska it is assumed that comparable conditions for growth occur somewhat later, although no new data justifying this were obtained. In order to show the effect of vertical position upon growth, means of the absolute increase per thirty days in the shell dimensions were plotted as a function of intertidal height (Fig. 4). This plot is presented as it does not seem justified to determine a mean instantaneous relative growth rate value (to be discussed later) for the entire growth period, and the data obtained by Coe and Fox (1942) were desired for comparison.

Big Rock mussels were initially marked and measured April 13, 1952. A second series of measurements was made May 25, 1952 and the last series, November 1, 1952. Mussels were measured in 1954 on the following dates: April 24, May 22, July 1 and July 30. Mussels at Mount Edgecumbe, Alaska were measured on the following dates: June 26, July 24 and September 4, 1952.

Measurements of the shell were used. Individuals were marked by filing an identification mark on the shell and were then allowed to remain in the mussel beds for the period of study. Particular effort was made not to disturb each mussel any more than was necessary. A firm attachment by the byssal threads was confirmed, or the animal was discarded initially. Dial calipers were used for the measurements, which had an accuracy of ± 0.1 mm. The three shell measurements used were the greatest shell length, width and height, except that length was not obtained for the 1952 sample from southern California. About two hundred individuals were measured in Alaska, and a like number in California.

To compare rates of growth of an attached species from different localities, it is necessary to locate groups of animals that occupy approximately comparable intertidal positions, with respect to wave action, degree of tidal exposure, compactness of the beds and size of animals that compose the bed. These parameters were difficult to measure, and assurance of ecologically similar conditions could be made only in a subjective way. The mussel beds at Mount Edgecumbe, Alaska and Big Rock, California were exposed directly to the open surf; the size of the animals was about the same (southern populations were somewhat larger); the mussel beds at the two localities were about the same in numbers of individuals per given area. On the causeway at Mount Edgecumbe, which consisted of a constructed road that served to connect several islands, and projected into the open ocean, the ecological distribution of *Mytilus californianus* was somewhat different from that observed at Big Rock. The intertidal zone is essentially vertical, and the gently sloping rocky coast so characteristic of southern California is lacking. Hence, it was necessary to attempt to locate mussel beds on a nearly vertical substratum that would be comparable, intertidally, to mussel beds in the south that had much greater horizontal distribution. The mussels in southern California were located at the 3.0 ft. (± 0.5 ft.) and 1.0 ft. (± 0.5 ft.) tide level and those in Alaska, at the 5.5 ft. (± 0.5 ft.) level (zero datum level is mean lower low water). The lower figures demark the lower level of vertical distribution of this species at the respective localities. It should be noted that at both localities populations of *Mytilus californianus* were distributed vertically in the intertidal to well-delineated levels, below and above which the populations were essentially non-existent. This does not take into account subtidal populations recently located on the southern California coast (Berry, 1953; Rao, 1953). Values given elsewhere in the text for hours and percentage exposure per week at these levels were calculated from Ricketts and Calvin (1952, Fig. 112).

Under field conditions the problem of recovery of marked animals was serious. Within one month there may occur a great deal of settling of barnacle larvae, young mussels and seaweed, thus obscuring marked animals. Some individuals moved along or within the bed. This movement may be over the surface of the bed, or, as in many cases, mussels moved vertically from the surface of the bed several inches down to the rock substratum. One month following a series of measurements, about 60% recovery was realized and two months later, approximately 40% of the remainder. At lower intertidal levels in southern California about 80% returns were obtained over the period of four months. It is assumed that the losses were non-selective with respect to the size of the animals.

METHOD OF ANALYSIS

As yet no adequate formula has been propounded to describe all phases of growth in any animal (Weymouth, McMillan and Rich, 1931; Huxley, 1932; Brody, 1945). For this material the most suitable expression is the instantaneous (true) relative growth rate $(k) = \frac{dL/dt}{L}$. A discussion of the significance and justification for using this method will be found elsewhere (Dehnel, 1955). The

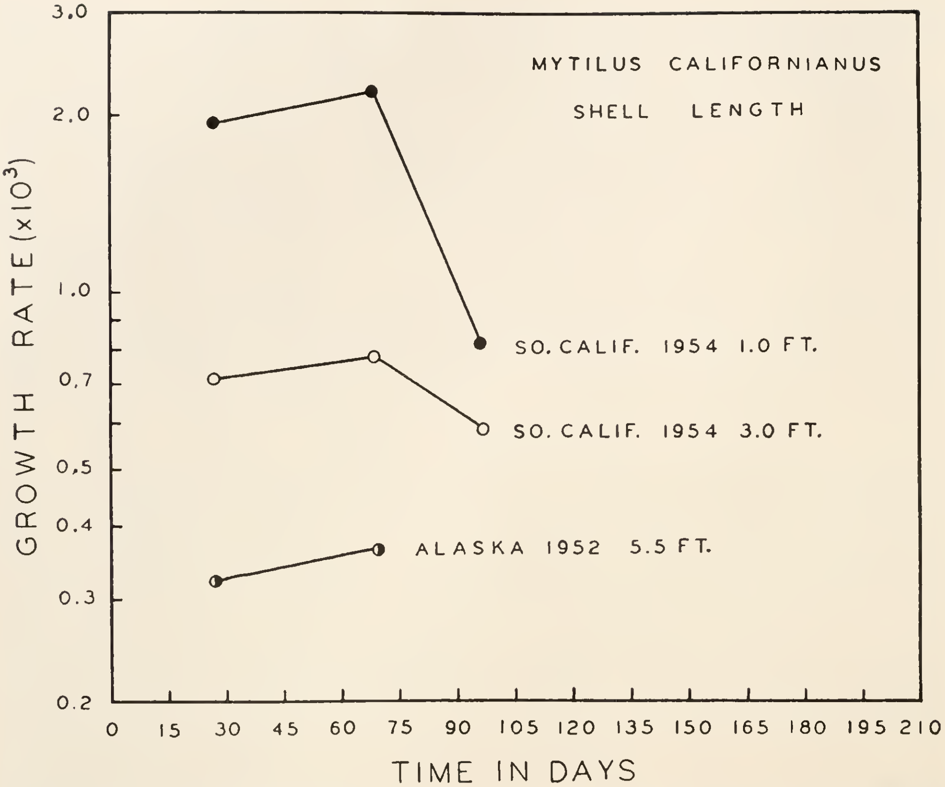

FIGURE 1. A comparison of the rate of growth in length of shell in populations of *Mytilus californianus* from Mount Edgecumbe, Alaska at one intertidal level, and Big Rock, California at two intertidal levels. Each point represents the mean k value for a sample of fifty or more individuals. The initial value is plotted on the day at which the second measurement was made.

instantaneous relative growth rates of the length, width and height of the shell were plotted as a function of time (Figs. 1, 2, 3). The initial value is plotted on the day at which the second measurement was made.

The means of the initial length, width and height of the shell are about 25% greater for the southern California mussels sampled than for northern ones. Presumably, similar size in latitudinally separated populations of a given species indicates different chronological ages, the northern ones being older (Wimpenny, 1941). Hence, samples of the northern and southern populations under consideration should be approximately the same age. A small initial size was chosen, as growth rates are more rapid in young animals.

RESULTS

Growth rate in latitudinally separated populations

When the instantaneous relative growth rate of the length of the shell is plotted as a function of time (days), it is seen that the rate of linear size increase of this dimension is greater in the population from southern California at the 3.0 ft. level (Fig. 1) than in the Alaskan one (5.5 ft. level). The rate of the northern sample is about 45% of the southern one, initially, but the slopes of the two curves are nearly parallel. The mean absolute increase per thirty days for the southern California sample over the period from April 24 to July 30, 1954 was 0.96 mm.; for the smaller mussels from Alaska (June 26 to September 4, 1952), the increase was 0.33 mm. In the middle part of the summer, the rate for the southern sample decreases, but is still growing at a rate greater than the one from Alaska, as seen from an extrapolation of the northern curve.

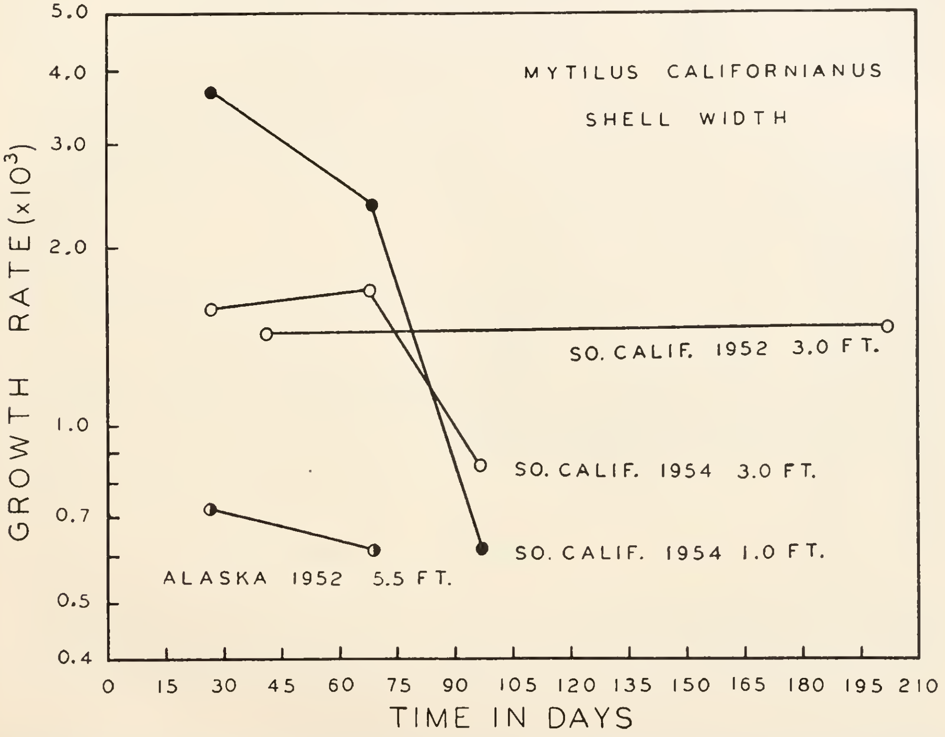

FIGURE 2. A comparison of the rate of growth in width of shell in populations of *Mytilus californianus* from Mount Edgecumbe, Alaska at one intertidal level, and Big Rock, California at two intertidal levels. Each point represents the mean *k* value for a sample of fifty or more individuals. The initial value is plotted on the day at which the second measurement was made.

Also, the rate of linear increase in shell width is greater in the population from the 3.0 ft. level from southern California when compared with the population at the 5.5 ft. level from Alaska (Fig. 2). The rate of the northern sample, again, is about 45% of the southern one. The initial rates of the two samples from southern California are very similar. The 1952 sample shows no decline in rate due to the lack of intermediate points. For the years 1952 and 1954 the mean absolute increase per thirty days was 0.90 mm. and 0.81 mm., respectively. The mussels from Alaska showed a 0.30 mm. increase for the same length of time.

With regard to shell height the growth rate for the southern sample is greater (Fig. 3). Comparing the 1954 sample from southern California (3.0 ft.) with the Alaskan one, there is essentially no difference between the initial rates. However, subsequent segments of the curve show that the northern sample is decreasing its rate to a greater extent. The 1952 sample from southern California at the same intertidal level has a rate which is about 46% of the Alaskan group. The increase per thirty days for shell height was 0.85 mm. in 1952 and 0.69 mm. in 1954 for the sample from southern California, and 0.34 mm. for the northern one.

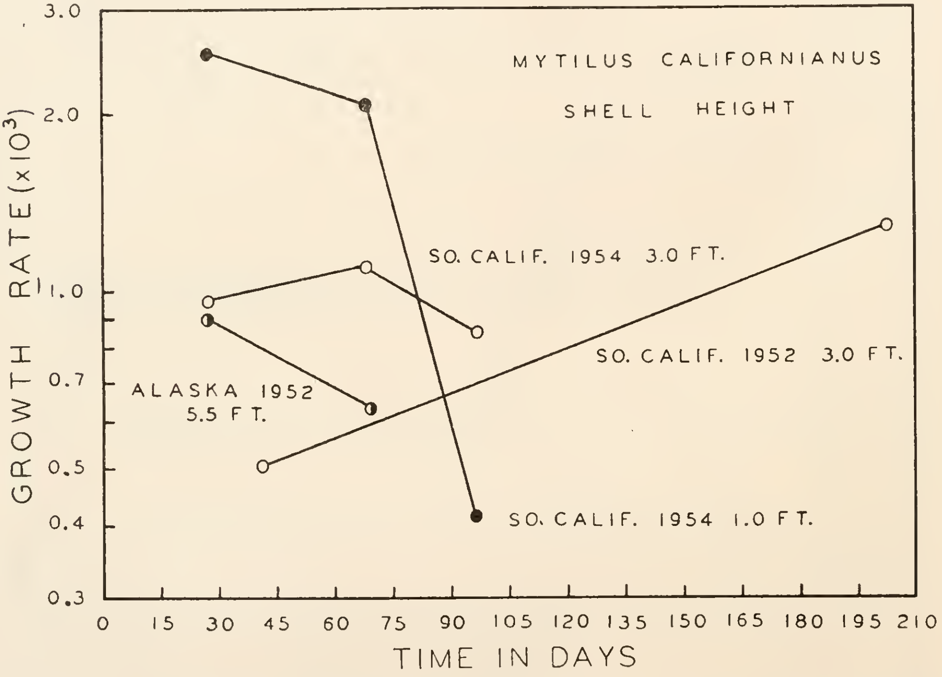

FIGURE 3. A comparison of the rate of growth in height of shell in populations of *Mytilus californianus* from Mount Edgecumbe, Alaska at one intertidal level, and Big Rock, California at two intertidal levels. Each point represents the mean *k* value for a sample of fifty or more individuals. The initial value is plotted on the day at which the second measurement was made.

It is evident from Figures 2 and 3 that the line drawn between the two *k* values (which represents the second and third measurements, May 25 to November 1, 1952) of both width and height of the populations from southern California (3.0 ft.) does not describe the conditions of linear increase in shell dimensions because of a lack of intermediate points. As a result, a more refined comparison might be made between the first two points on the curve (April 13 to May 25, southern California; June 26 to July 24, Alaska). Absolute increase in shell width for southern mussels was 0.86 mm. per thirty days, and for northern ones 0.32 mm. Increase in shell height for southern animals was 0.64 mm. per thirty days, and 0.43 mm. for northern ones. These latter values differ somewhat from the initial ones, because of the inclusion of more animals recovered at the second measurement. Relative rate (k) values have been determined for each individual of both populations, and then a mean rate calculated. Standard deviation for each rate was obtained, and a test was made to determine the significance of the difference between the two means (Alaska and southern California) when the population standard deviations are known. The mean rates for shell width are as follows: Alaska, $k = 0.00081$ (± 0.00055); southern California, $k = 0.00170$ (± 0.00166). The difference between these two means is significant ($P < 0.002$). Values for shell height are as follows: Alaska, $k = 0.00111$ (± 0.00083); southern California, $k = 0.00142$ (± 0.00107). The difference between these two means is insignificant ($P = 0.100$); such differences could occur by chance alone 10% of the time. A comparison of the mean rates (k) for length of shell between Alaskan and southern California mussels (3.0 ft.) shows them to be statistically significant ($P < 0.002$).

Comparison of the absolute increase per thirty days and/or the relative rate of increase of linear size between the 1952 and 1954 samples from southern California at the 3.0 ft. level is of value. This comparison points out the similarity of linear increase for two years in this population and is suggestive that the values obtained are characteristic of rates of increase of animals from this locality. These values also suggest that sea water temperatures were similar, which they were, and that the amount of plankton available was similar.

From these data, it would appear that the rate of size increase in shell length and width is greater in the sample of mussels from southern California than in that from Alaska, but the increase in shell height, though greater, cannot be said to be significantly different on these data. This means the shells are also growing differently shaped in the two populations.

Growth rate in vertically separated populations

During the spring and summer of 1954 not only were mussels studied at the 3.0 ft. tide level, but also at the 1.0 ft. level. The purpose of this was to determine the effect of intertidal height on the rate of linear increase of shell dimensions in this species. Reference to Figure 4 shows the differences in absolute increase in all shell dimensions per thirty days as it varies with tidal height. Data from Coe and Fox (1942) are included on this graph in order to illustrate the increase in mussels from La Jolla, California that were totally submerged. A comparison between populations located at the 1.0 ft. and the 3.0 ft. tidal levels from southern California indicates the great effect submergence has upon growth in this filter-feeding invertebrate.

Inspection of the data shows that the absolute increase in shell length at the 1.0 ft. level is over two times that at the 3.0 ft. level (2.3 mm./0.96 mm.), for a thirty-day period. With respect to width, the difference is not so great (1.3 mm./0.90 mm., 1952; 0.81 mm., 1954). An average of the last two values shows the mean increase of the higher level to be about 66% of the lower one. Absolute increase in height is about the same as the width relationship (1.2 mm./0.85 mm., 1952; 0.69 mm., 1954). An average of these values shows the 3.0 ft. animals to increase about 64% of the 1.0 ft. mussels.

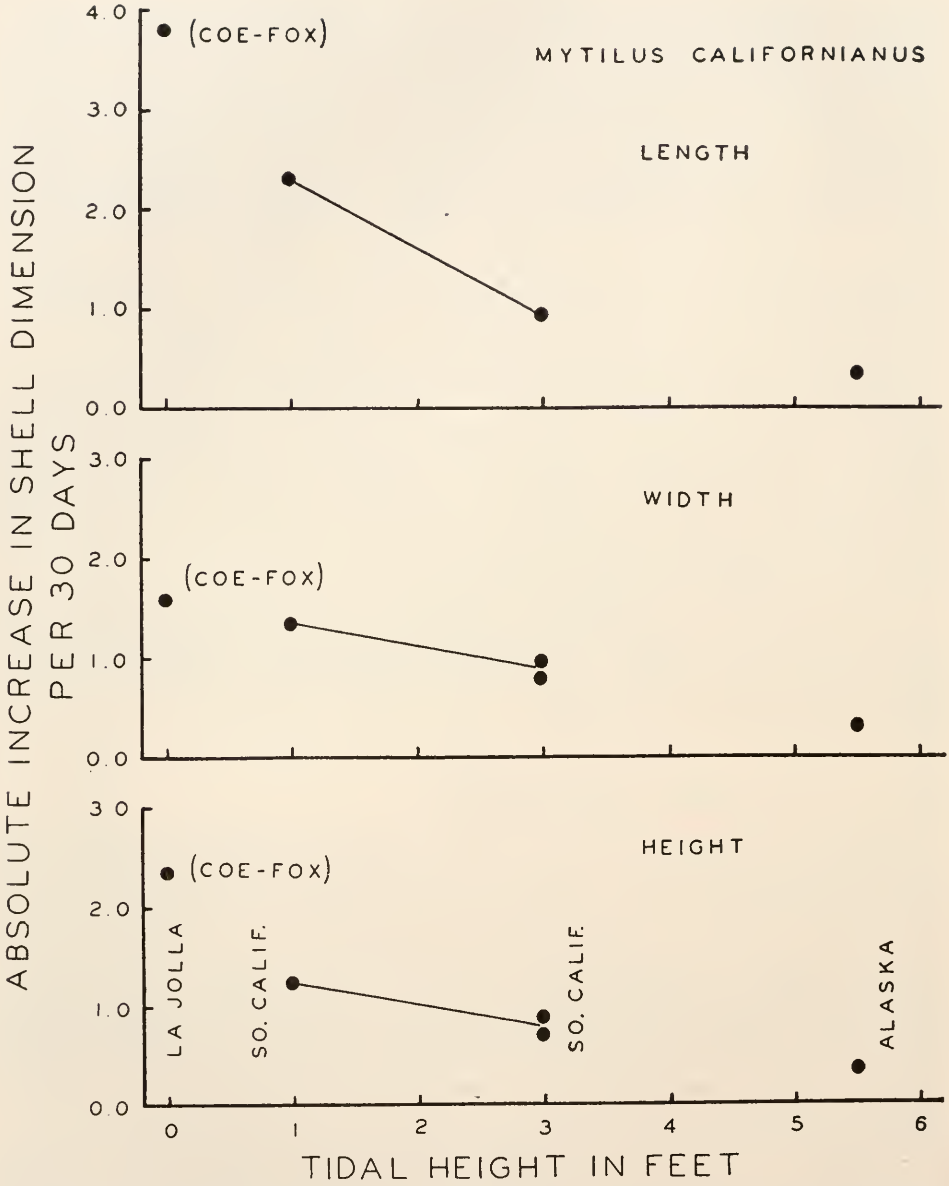

FIGURE 4. A comparison of the absolute increase in shell dimensions per thirty days as a function of intertidal height in populations of *Mytilus californianus* from La Jolla, California, Big Rock, California and Mount Edgecumbe, Alaska. Data for La Jolla mussels were obtained from Coe and Fox (1942, page 7, Table 1). Each point represents the mean increase for a sample of fifty or more individuals.

DISCUSSION

It would be well to mention at the outset that the data presented by Coe and Fox (1942) for growth of mussels at La Jolla, California, under totally submerged conditions are in relative agreement with rates obtained at Big Rock, California, at the 1.0 and 3.0 ft. tide levels. In their paper, Coe and Fox (1942) present length measurements, and ratios from which width and height measurements may be obtained. Their ratios of $W/L = 0.37$ and $H/L = 0.5$ are not in agreement with the ones from Big Rock at either tidal level. However, there is no reason to believe that populations of mussels under entirely different ecological conditions should have the same growth ratios as ones submerged completely. Such mussels would conceivably compare only with subtidal populations of mussels, and even then growth might be much different.

The most obvious difference between populations of mussels from Big Rock at the 3.0 ft. and the 1.0 ft. tide levels is the degree of submergence per given period of time. Mussels located at the 3.0 ft. level were submerged about 58% of the time (14 hours per day), and ones from the 1.0 ft. level were submerged about 88% or twenty-one hours per day. It may be assumed that mussels obtain food in proportion to their access to it, *i.e.*, to their time of submergence. Rao (1954) has adduced good evidence for this. Then it may be expected that the food intake of the 1.0 ft. mussels compares with that of the 3.0 ft. ones as 88:58 or 100:66. If it is assumed that no difference existed in available food material per liter of sea water between the two populations at Big Rock, the difference between rates of linear increase in these two populations is partly explained.

The significance of vertical distribution as it affects rates of linear increase in species such as *Mytilus californianus* becomes apparent when mussels from southern California and Alaska are compared. Previously, it was stated that one population of Big Rock mussels was located at the 3.0 ft. tide level. These animals are able to filter approximately fourteen hours per day. Mussels from the population studied in Alaska were located at the 5.5 ft. tide level. They were able to filter about ten hours per day. The Big Rock mussels are submerged 1.4 times longer per twenty-four hours than the Alaskan ones. Even though the degree of submergence differs in the two populations, it is felt that mussels located at the 5.5 ft. level in Alaska are ecologically equivalent to ones from Big Rock at the 3.0 ft. tide level.

From values given by Rao (1953) pumping rates as a function of weight of soft parts for mussels from different latitudes may be determined. Weights of animals used were nearly the same (northern mussels have heavier shells relative to body weight), and by taking into account latitudinal acclimation, Big Rock mussels (16° C.) filter about two liters per hour, those from Alaska (10° C.), about one liter per hour. If the available organic matter per liter of sea water for northern and southern waters is the same, Big Rock mussels would filter $1.4 \times 2 = 2.8$ times as much food as the higher intertidal and only partially acclimated Alaskan sample. The relative growth rates (k) were found to be in the ratio 2:1 for length, 2:1, width and 1.3:1 for height. We may attempt to estimate the expected rate of linear growth in shell dimensions of Alaskan mussels if they were submerged to the same extent as the ones from Big Rock located at the 3.0 ft. level. Volume change must be calculated and this converted into linear increase. The ratio of food obtained is 2.8 and its cube root is 1.41. The value thus found gives the ratio of expected fourteen hour to observed ten hour increase in length, width or height for Alaskan mussels. Also, this value (1.41) may be compared with the growth rate ratios given above. Considering the roughness of the estimate, the agreement is good and suggests that there is acclimation of growth rate.

If the interpretation presented is valid, and part of it is based upon the assumption and belief that the northern mussels are ecologically equivalent to lower intertidal southern ones, it could then be stated that even though relative rates in a population of *Mytilus californianus* from southern California are greater, mussels from Alaska, if chosen from a level with the same periods of submergence, would grow at approximately the same rate in spite of the lower temperature.

SUMMARY

1. Rates of growth have been studied in populations of *Mytilus californianus* collected from southern California and southeastern Alaska. As nearly as possible natural rates of growth have been determined from populations in the field.

2. Length, width and height of the shell are used as criteria for determining rates of growth in this species. Comparisons of rates of growth between northern and southern populations are made by the use of relative rate (k) values.

3. Mussels were measured at the 1.0 ft. and 3.0 ft. tidal levels in southern California and the 5.5 ft. level in Alaska.

4. Rates of growth of *Mytilus* are found to be greater in the sample from southern California, comparing the 3.0 ft. level, than in that from Alaska. However, it was found that the southern California population is submerged for a greater period of time per week. The greater period of submergence and the greater weight of soft parts for a given shell size, recently shown by Rao (1953) for the southern population, mean a greater food intake in spite of acclimation of pumping rate. Attempts to correct for these factors suggest that the actual discrepancy in intrinsic growth rate is small or absent. In particular, Rao's findings give reason to believe that the heavier bodied southern mussels lay down no more shell length per unit of body weight than comparable northern ones.

5. Comparison of relative rates of growth in vertically separated populations of this species shows a large positive effect of submergence upon rate of increase of shell dimensions.

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THE MIGRATORY HABITS OF THE MARINE GRIBBLE LIMNORIA TRIPUNCTATA MENZIES IN SAN DIEGO HARBOR, CALIFORNIA[1, 2]

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The isopod gribble *Limnoria* is a serious wood-boring pest in nearly all tropical, temperate, and boreal harbors where contamination has been established and where salinities are not markedly reduced by influx of fresh water. Typical examples of the ravishes of one or more species of this borer are offered by the findings of the San Francisco Bay Marine Piling Committee (Kofoid and Miller, 1927). During that survey only *Limnoria lignorum* (Rathke) was recognized, but Holthuis (1949) has shown that the species *L. quadripunctata* described by him as new was present in the area and had been confused with *L. lignorum*. Actually two species exist in San Francisco Bay, neither of which is *lignorum* (Menzies and Mohr, 1952).

Examination of collections from the San Diego region and elsewhere in California led Menzies (1951) to establish yet another species, *L. tripunctata* which has hitherto also been confused with *L. lignorum*.

Along the west coast of North America the geographic ranges of the three species of wood-boring *Limnoria* have been established as follows (Menzies, in manuscript): *Limnoria lignorum* (Rathke); Alaska to Point Arena, California. *Limnoria quadripunctata* Holthuis; California (41° N) to San Diego Bay. *Limnoria tripunctata* Menzies; warm parts of San Francisco Bay to Mazatlan, Mexico.

The seasonal activities of *L. lignorum* were studied at Friday Harbor, Washington, by Johnson (1935) who found that that species undergoes a pronounced seasonal swimming migration at which time new wood is attacked mainly during early spring. Sömme (1940) confirmed this type of activity for the same species in Norway and reported that a survey by Kramp in 1927 showed similar activity in Danish waters. Kramp's report, published in a Danish engineering journal, has escaped the attention of biologists in other parts of the world. He gives no figures of relative abundance or intensity of attack by *Limnoria lignorum* on new test blocks but states that at Hirtshals, Denmark, there was a pronounced maximum of attack in April. He concludes that the attacks result from periodic swarming of mainly adult individuals. Following the April swarming, the attacks occur in diminishing intensity during the summer.

It is of practical interest to know to what extent the findings relative to the northern species *L. lignorum* can be generally applied to the other species. With this in mind, the present study was designed to test *L. tripunctata*, a species of world-wide distribution in southern waters and the only one found in San Diego Bay.

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PROCEDURE

As a means of determining the migratory habits of *Limnoria tripunctata,* wooden test blocks were suspended freely in the water in the immediate vicinity of piling known to be heavily infested with *Limnoria.*

The test blocks consisted of 5 × 8 × 1 inch Douglas fir which provided about 106 sq. in. (684 cm.^{2}) of surface that could be examined by means of a low-power binocular microscope. After having been soaked in sea water free of borers for a period of 14 days, the blocks were attached to a rope line or stainless steel wire which was suspended from the pier and held taut by a concrete weight attached to the lower end, such that the weight hung free of the bottom. Test blocks thus rigged were located about one foot from infested piling so that they could be reached only by swimming migrating animals.

Two test stations were established in San Diego Bay: Station "A" at the U. S. Naval target repair base at Point Loma and Station "B" at the Johnson and Western Pier well within the bay near the Coronado ferry landing. Owing to reconstruction and repair there was some interruption at each station. The vertical position of the blocks and approximate days of exposure for each are given in Table I.

TABLE I

Position and exposure of test blocks

Station A

Aug. 12, '52–Aug. 13, '53		Aug. 13, '53–Feb. 15, '54		Apr. 14, '54–Mar. 14, '55	
Position of blocks	Days of exposure	Position of blocks	Days of exposure	Position of blocks	Days of exposure
Floating*	60				
Floating	30	Floating	30	Floating	30
6 ft. from bottom	30	6 ft. from bottom	60	6 ft. from bottom	60
4 ft. from bottom	30	4 ft. from bottom	90	4 ft. from bottom	90

* Zero tide datum is approximately 14.6 feet above bottom. The mean tidal range was approximately 4 feet.

Station B

Aug. 15, 1952–Aug. 13, 1953		Feb. 15, 1954–Mar. 14, 1955	
Position of blocks	Days of exposure	Position of blocks	Days of exposure
Floating**	30	5 ft. from bottom	30
2 ft. from bottom	30	4 ft. from bottom	60
		3 ft. from bottom	90
		2 ft. from bottom	120

** Zero tide datum is approximately 9 feet above bottom. Tidal range same as for Station A.

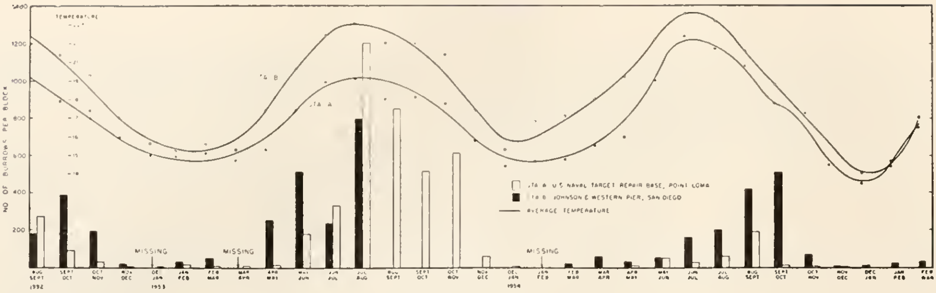

FIGURE 1. *Limnoria tripunctata.* Seasonal attack on test blocks exposed for successive 30-day intervals at Stations A and B, San Diego Bay from August, 1952 to March 14, 1955. Seasonal temperature included.

The intensity of settlement or attack on the blocks for a given period is taken to represent the intensity of migration for the period. It is determined by counting the number of burrows in the surface of the wood. Usually there are two animals, male and female, in each of the deeper burrows.

Surface temperature of the water was taken once each month at each station except for the period 15 August 1953 to 7 May 1954 when a continuous temperature recorder was in operation near Station A. Salinities in San Diego Bay fluctuate but little. The mean annual value is about 33.6‰.

Requirements for a study of this nature are: (1) nearby ample source of gribble infestation; (2) little or no interference resulting from repair or removal of piling; (3) safety from ship action, heavy seas or pilferage; (4) absence of adverse conditions due to low dissolved oxygen or low salinity.

These requirements were well met but for the already mentioned changes due to pier repair.

RESULTS

Seasonal Migrations

An examination of the severity of attack on the test blocks reveals that migration occurs to a greater or lesser degree throughout the year, but as in *L. lignorum,* there was each year a period of pronounced increase in migratory activity. In migrating the animals swim freely and a special preliminary study designed to reveal diurnal behavior in nature indicates that the greatest swimming activity takes place at night.

In Figure 1 is shown graphically the monthly seasonal fluctuations in migration between Aug. 12, 1952 and March 14, 1955 inclusive. The data used in compiling the figure were derived from the blocks submerged for successive 30-day periods with omissions as noted. Other periods of submergence, especially the 60-day periods, provided corroborative data on season of attack (Fig. 5) but the longer periods of submergence were designed mainly to provide information on rate of growth, sexual condition and reproduction.

The test blocks that were in place for 30-day periods beginning August 12, 1952, apparently caught mainly the declining portion of that year's migration.

In 1953 at Station A there was a period of active migration which extended from about the middle of May to the middle of November with a maximum during July–

August. This was also the peak 30-day period for Station B. This station was abandoned in August, 1953 when it was apparent that both stations at the various depth levels were showing a similar migratory picture; however, it was re-established when the reconstruction of piles at Station A forced a temporary abandonment of that station in January, 1954. By April, 1954 both stations were again studied until the completion of this phase of the work in March, 1955.

From the time of re-establishing of Station A in April, 1954, the attack on the blocks was relatively light but with a definite maximum occurring this time in August–September. The lighter attack may be attributed to a diminished source of infection as a result of replacement of infested piling, etc., with new pressure-treated creosoted timbers which left only a nearby infested stub and doubtless other undetected submerged wood, and some more distant infested structures. This also explains the low migratory figure shown in Figure 5 for the second year.

The main season of attack at Station B appears to be during August and September after which it diminishes rapidly to the low winter level.

The seasons of active attack on the blocks roughly coincide at the two stations, but it appears to have continued somewhat later at Station B in 1952 and began slightly earlier in 1953. The same trend is suggested in 1954. This difference may be correlated with the temperature. From Figure 1 it will be noted that a higher temperature prevailed throughout the year at Station B.

It has commonly been reported that the attack by *Limnoria lignorum* is not so great on floating or shallowly placed test blocks as it is on deeper blocks or near the bottom (Johnson and Miller, 1935; Sömme, 1940; Black and Elsey, 1948). This may be a factor of considerable importance in the relative rate of deterioration of floating or driven structures. However, our data from *Limnoria tripunctata* indicate that it shows no marked preference with respect to depth of settlement.

In order to better understand the migratory activity of *Limnoria* in light of its population biology an analysis was made of the seasonal composition of the population from the piling and from the test blocks. For this purpose the animals were studied as to size and, in the older specimens, also as to sex and reproductive condition.

Accurate measurements of the body length of *Limnoria* are usually difficult to obtain because of the natural tendency of the animals to assume a strongly bent position or for the segments to become more or less telescoped depending upon the type of preservative used or upon other factors such as ovarial development or injury. A practical substitute is therefore here made by using the pleotelson width at its widest point as an index of size. This has the advantage that we here deal with a flat rigid structure (the last segment of the body) that is not readily distorted. It has the further advantage that the size of animals in shallow burrows may often be determined without removing them from the burrow, and also the size of exuviae can be determined by this one fragment of the generally disarticulated cast.

Ten size categories can be established more or less clearly based on the width of the pleotelson.

In the literature dealing with *Limnoria*, the size index given is always in length; hence it will be useful here to provide Table II giving conversions of pleotelson widths to body lengths for the ten groups.

TABLE II

Limnoria tripunctata. Pleotelson width to body length. 180 animals measured

Size class	Mean pleotelson width, mm.	Body length, mm.		Remarks
		♂	♀	
1	0.24–0.26	0.8–1.2		Young lacking seventh pair of pereiopods
2	0.28–0.33	1.0–1.6		
3	0.35–0.40	1.1–1.6		Young with underdeveloped seventh pair of pereiopods
4	0.42–0.46	1.5–2.2	1.5–1.5	Sexual maturity reached by males
5	0.48–0.53	1.7–2.2	1.9–3.0	Females develop oostegites
6	0.55–0.60	2.1–3.0	1.8–3.0	Females become gravid
7	0.61–0.67	2.0–3.3	2.6–3.5	Includes majority of reproductive individuals, especially the females
8	0.69–0.74	2.5–3.4	3.0–3.8	
9	0.76–0.81	2.5–3.4	2.7–3.5	
10	0.82–0.86	2.5–3.2	2.5–3.8	Only few reach this size

An analysis of the population composition of a one-month test block and of old piling on the same date are given respectively in Tables III and IV. The results are quite typical, but for piling populations, there may be considerable variation in samples of one square inch in size taken adjacently in the same pile; hence, in most instances, counts were made in duplicate or triplicate.

A comparison of the percentage composition of the populations shown in Tables III and IV reveals that over 97% of the migrants are sexually mature animals of fairly large size, whereas on the piling, only about 28% are sexually mature, over 50% are newly hatched or immature animals, and 19% intermediate.

Analyses of piling samples taken monthly are given in Table V where the percentage composition of size classes are given and the mean calculated for three-month periods. Thus the variations are smoothed out to give seasonal means for

TABLE III

Limnoria tripunctata. Migrant population, Station A, Sept. 13, 1952. Numbers in size class, maturity, sex and per cent of total population (164) in test block of each month exposure

Size class	Immature	♀ (?) Young	♀ with oostegites	♀ gravid	♂
3	1				
4		2			1
5		1			16
6					12
7			21	7	25
8			12	10	11
9			18	11	10
10			2	2	2
% of Total	0.60	1.81	32.29	18.26	46.90

TABLE IV

Limnoria tripunctata. Piling population, Station A, Sept. 13, 1952. Numbers in size class, maturity, sex and per cent of total population in one square inch sample of infested wood with a total of 432 animals

Size class	7th pereiopod lacking	Immature	♀ (?) Young	♀ with oostegites	♀ gravid	♂
1	83					
2	36	39				
3		69	39			
4			39			8
5			5	4		10
6				10		7
7				7	13	23
8				8	10	7
9				4	7	4
% of Total	27.54	24.99	19.19	7.62	6.93	13.64

spring, summer, autumn, and winter. From this it appears that the piling population remains rather constant in its composition with some preponderance of the youngest class during the migratory months (June–November) and of the middle group during the winter (December–February) and relatively more adults in the spring (March–May) prior to heavy migration.

In the test block population presented in Table III, there is a slight predominance of females over males. It appears, however, from analyses of many block

TABLE V

Limnoria tripunctata. The mean percentage of size composition for monthly and seasonal periods for piling populations, 1952–1953

Size class	Spring			Seasonal mean	Summer			Seasonal mean
	March	April	May		June	July	August	
1–3	16	65.5	34	38.5	31.5	48	44	41.3
4–7	53.8	19.5	40.3	38	48	43.5	35	42.3
8–10	33.7	20	29	27.5	20.5	8.5	21	16.7
No. examined	403	783	792		545	449	300	

Size class	Autumn			Seasonal mean	Winter			Seasonal mean
	Sept.	Oct.	Nov.		Dec.	Jan.	Feb.	
1–3	69	34	47.5	50.3	17	53	33	34.3
4–7	16	51	40.3	35.8	63.5	34	54	50.7
8–10	15	15	12.3	14.1	19.6	13	13	15.2
No. examined	449	328	1282		557	153	44	

and piling samples, that the ratio of males to females on the piling is on an average about 1 to 1 for sexually mature specimens, whereas on test blocks it is often 1.3 to 1. This suggests that the males have a greater tendency to migrate than do the females, especially the younger females. Mating occurs after migration, the females becoming gravid some time after they have become established on the test block. This is evidenced by observations that (1) gravid females were found only in the longest, hence oldest burrows of the thirty-day test blocks, (2) here they occupied the blind end of the burrow while the male occupied the open end, (3) no gravid females were observed to occupy a burrow singly. This is in accord with the conclusions regarding the breeding habits of *Limnoria lignorum* (Kramp, 1927; Johnson, 1935; Sömme, 1940). Further evidence substantiating the opinion that migration precedes fertilization is found in Menzies' (1954) report which shows that females lack a sperm storage organ and the sperm produced by the male are relatively few in number and not grouped into spermatophores.

Sperm were found only in the oviduct of females which were moulting prior to deposition of eggs into the brood pouch. None were found in virgin or gravid females. Copulation probably precedes each brood production.

Factors Influencing Migration

Temperature

As shown in Figures 1 and 5 for *Limnoria tripunctata* and Figure 2 for *L. lignorum,* there is a certain correlation between the water temperature and the intensity of migration as indicated by severity of attack on the test pieces. The figures do, however, show a sharp difference in the maximum migratory period of the two species as related to the temperature curve and season of the year. *L. lignorum* reaches a peak of migratory activity on a rising temperature whereas *L. tripunctata* follows more nearly the seasonal trend of temperature with some tendency to lag behind. However, it should be pointed out that while the above appears to be typical for *L. lignorum,* at widely separated areas, data from Tromsösund, Norway, show a period of greatest migratory activity on a falling temperature. Sömme (1940) concluded that the season of migration varied with hydrographic conditions; hence each locality requires special investigation to determine the season of maximum attack on new wood. In a survey designed mainly to test the depth of attack in British Columbia waters, Black and Elsey (1948) indicate some variation in season of attack by *L. lignorum.* However, their data show that in areas such as Williams Head, Bentinick Island, and Esquimalt, which are not seriously affected by dilution, the main attack was during spring. The data used in Figure 2 A and B are derived from analysis of test blocks kindly provided us by the William F. Clapp Laboratories.

These differences in the two species may arise from specific differences in temperature requirements. Temperature *per se* may, however, not be the immediate cause of migration in either species of *Limnoria.* It is perhaps mediated through its effect on the rate of other biological activities such as growth, attainment of sexual maturity, and reproduction. Seasonal increases in these rates must lead to population pressure which is more acute at some seasons than at others.

Population pressure

In the study of *L. lignorum* at Friday Harbor (Johnson, 1935), it was hypothesized that migration results from overcrowding and approach of sexual maturity. Overcrowding is, of course, closely dependent upon the nature and availability of food supply and living space. *Limnoria,* like other wood borers, is unique in that it eats the substratum on which it lives. Limits to size and growth of the population are therefore set by the area of available wood. When conditions become intolerable due to population pressure, either a migratory movement must take place or the ratio of reproduction and growth to mortality must change. That the first takes place, we know, and there is some evidence also of the latter. The rate of growth, age of attainment of sexual maturity, and fecundity are then of prime importance in an understanding of migration.

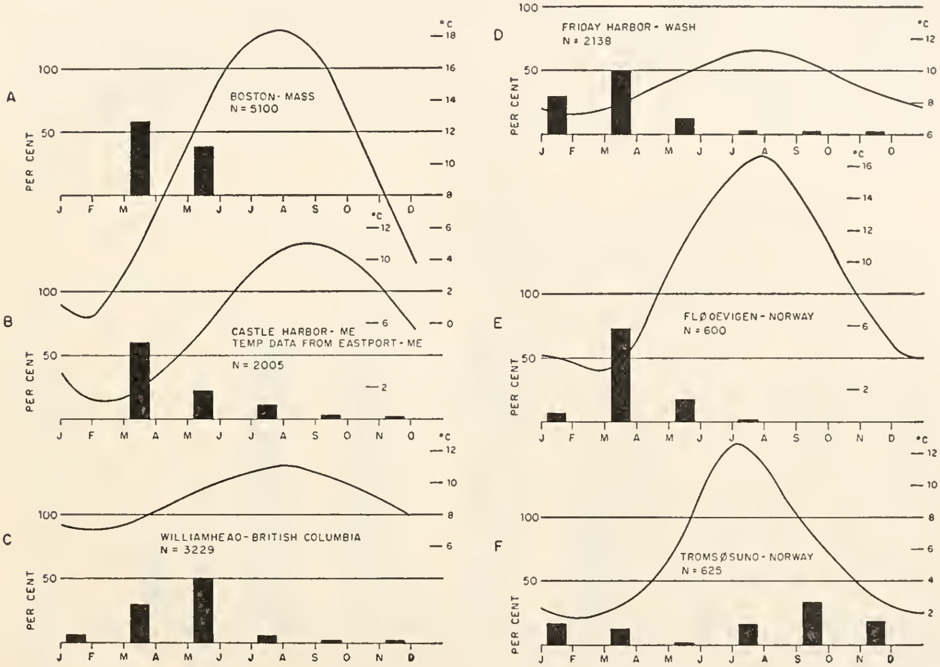

FIGURE 2. *Limnoria lignorum.* Percentage of seasonal attack in bimonthly periods in various localities. A and B data from William F. Clapp Laboratories; C from Black and Elsey, 1948; D from Johnson, 1935; E and F from Sömme, 1940. N = Yearly sum of animals on test blocks.

Fecundity Related to Overcrowding

That there is a reduction of fecundity in a crowded population is indicated in Table VI. Here it is apparent that with increased size of the female, as shown by pleotelson width, there is also an increase in the number of eggs produced. This relationship is more evident for the test-block than for the piling population. It will be noted also that the brood size of piling populations remains approximately one-half the size of that of the migrant population irrespective of size increase in the female. Thus it appears that an increased population density influences the brood size so that the rate at which young are added to a dense population may be only about one-half that at which they are added to a sparse population with plenty of wood for expansion. However, even with this adjustment, the population pressure must increase as the living space diminishes.

Fecundity Related to Temperature

The rate of development of eggs in the ovaries appears to be temperature-dependent. Brood-bearing females were found at Station A on test blocks which had been submerged for 30-day intervals all months of the year except the winter. In July and August, 1954, a brood was produced and hatched in 30 days at a temperature near 20.8° C. Test blocks submerged for 60 days showed gravid females throughout the year and hatched young most months of the year except during the spring of 1953 and in January and February, 1954. Station B yielded similar results for the 30-day exposures except that a brood was produced and hatched in less than 30 days in April and May (mean temperature 19.5° C.). The deposition of eggs and hatching of broods did not occur within 60 days at Station A during the winter or spring months of 1953–54 when temperatures ranged from 14.8 to 15.8° C. This suggests that with a temperature difference of only 5° C., it takes about twice as long to produce and incubate young during winter as it does during the summer.

TABLE VI

Limnoria tripunctata. Comparison of brood size with size in females from old wharf piles and from 30-day test blocks

	Piling			Test blocks		
Size class	No./brood		No. gravid ♀♀ examined	No./brood		No. gravid ♀♀ examined
	Mean	Range		Mean	Range	
6	4.6	1–6	11	—	—	None found
7	5.8	1–12	25	8.9	4–18	35
8	4.0	1–12	28	10.8	1–18	79
9	5.1	1–12	34	12.4	1–22	95
10	7.0	1–12	3	10.5	4–21	21

It was observed that at least during the summer months, females with hatched young also had ripe ovaries and could incubate another brood soon after the emergence of the first batch from the pouch. Frequently young and their secondary or lateral burrows were observed in a burrow of a gravid female. In the test blocks exposed for three-month periods, the following maximum numbers of broods were observed: Station A, mid-November, 1953–mid-February, 1954 (mean temperature 14.8° C.) one brood; mid-July–mid-October, 1954 (mean temperature 20.6° C.) three broods; mid-August–mid-November, 1954 (mean temperature 17.4° C.) two broods; Station B, mid-February–mid-May, 1953 (mean temperature 18.6° C.) two broods. Using size as a criterion, it appears that migrant females may have produced one or two broods prior to migration. This is indicated in Figure 3 which should be coordinated with Table II showing that females may become gravid in size class 6. Females in size class 10, the largest, were found only in migrant populations. Although migrants must frequently land on already heavily infested wood, the relative absence of the large females probably indicates that few, if any, re-establish themselves in dense populations. Thus the implication is that migration is largely an urge to seek less crowded conditions. Analysis of the population forming the basis of Figure 3 shows also that the summer and fall gravid migrants average 0.04 mm. smaller than those of other seasons and the gravid females from piling average 0.05 mm. smaller, thus indicating that sexual maturity is reached at a smaller size (hence more rapidly) during the warmer months.

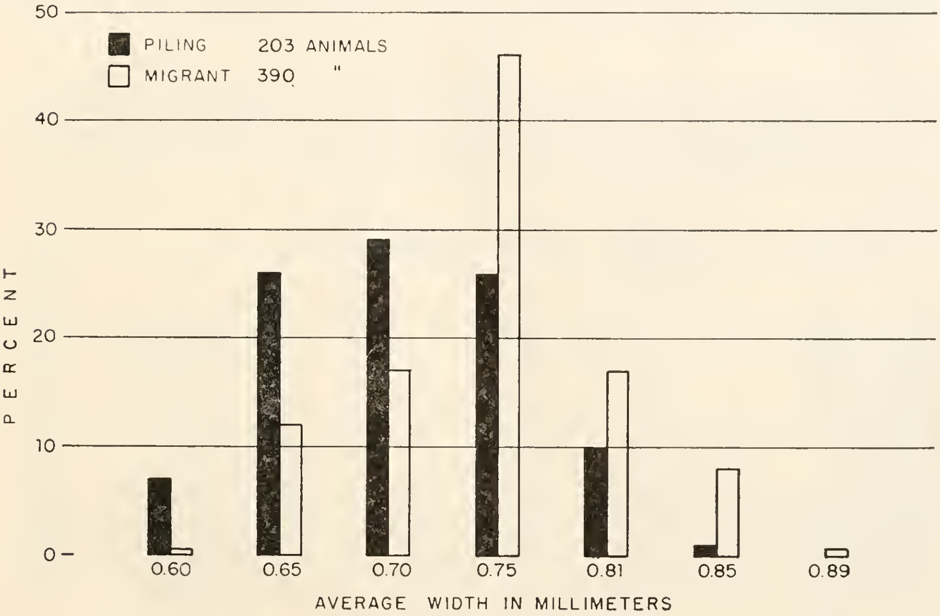

FIGURE 3. *Limnoria tripunctata.* Percentage size composition of gravid female populations.

That reproduction in *Limnoria* is influenced seasonally by temperature is further indicated by a study of the "gravidity" which may be defined as the per cent of gravid females among the adult females occurring at any time in a piling population of maximum density (Table VII).

From Table VII, it seems evident that gravidity for this species reaches its peak somewhere between 17° and 19° C., declining at temperatures above and below these values. Gravidity appears to double between 14° and 18° C. In laboratory cultures only 28% became gravid at 24° C. The broods of specimens kept at 34° C. deteriorated, suggesting that such high temperatures may unfavorably affect brood production. At Beaufort, N. C., Coker (1923) reported no gravid females at

10° C. Mawatari (1950) reports that temperatures of 2–6° C. are lethal to *Limnoria lignorum* (probably *L. tripunctata*).

In contrast the northern species *Limnoria lignorum* appears to reach its peak gravidity at about 10° C., based on data from Johnson (1935). These data (assuming a 50-50 ratio of males to females) indicate that with environmental temperatures from 7.9° to 11.1° C. the gravidity figures varied from 22% to 79%, whereas at 7.5° C., the lowest temperature encountered, it was down to 14%. Sömme (1940) reports that *Limnoria* ceases to produce eggs at temperatures of 0° C. and below. In experiments she observed that the brood of a gravid female kept at 24.7° C. failed to develop after five days; at 14.8° C. and 6.3° C. development was apparently normal but at the lower temperature about twice the time was required as at an average of 13° C.

TABLE VII

Limnoria tripunctata. Seasonal gravidity (per cent gravid females of sexually mature females, 50/50 sex ratio assumed) in piling populations, Station A, 1952–1953. Figures based on gross size-class analyses of two to three one square inch samples of wood per month

Month sample collected	Mature animals of both sexes examined	Total females	Gravid females	% Gravid	Temperature °C.	
					Range	Approx. mean
January, 1953	96	48	6	12	15.5–14.8	15
February, 1953	124	62	34	55	14.8–14.6	15
March, 1953	289	44	83	57	14.6–15.0	15
April, 1953	109	54	31	57	15.0–15.5	15
May, 1953	220	110	68	62	15.5–16.3	16
June, 1953	133	66	53	80	16.3–18.3	17
July, 1953	44	22	12	55	18.3–19.2	18
August, 1953	53	26	28	100*	19.2–19.3	19
September, 1952	121	60	42	70	18.5–17.4	18
October, 1952	57	28	17	60	17.4–16.2	16
November, 1952	154	77	55	71	16.2–15.4	16
December, 1952	104	52	2	4	15.4–14.8	15

* In this case there were obviously more females than males in the sample.

Individual and population growth

The habits and life history peculiar to *Limnoria* make it possible to determine the growth rates of individuals and to follow the development of one or more broods in nature. To accomplish this, the test block (habitat) must have remained in the water a sufficiently long time to allow the migrants to incubate their broods and for the young to grow, but not so long that young of a second generation or new migrants confuse the picture. Development is direct and when the young emerge from the brood pouch, they immediately construct their own burrows within the walls of the parent burrow. The old female followed by the male continues burrowing deeper into the wood, so that subsequent broods released are separated more or less clearly from the previously released brood.

Thus up to the time that they may desert their burrows or have burrowed too far from the parent burrow, the young produced on the block can readily be distinguished from young which might have migrated to the block. Even specimens which have become sexually mature, and no longer associated with the parent burrow can usually be distinguished from sexually mature young migrants to the blocks because they occur in significantly larger numbers than do the young (small) migrants.

Rate of Growth.

The growth rate of *Limnoria tripunctata* was determined by culturing individuals in the laboratory and from measurements of the growth of young on test blocks for two-, three-, and four-month intervals. The maximum growth rate seasonally as found in test block exposures is shown in Figure 4. An evident feature is the seasonal difference in growth rate of the young. Those emerging in winter require roughly twice as long to reach size class 5 (with 0.48–0.53 mm. pleotelson width) as do the spring young, and three times as long as the summer young. Thus increased crowding from growth of individual animals (distinguished from increased crowding resulting from addition of new individuals) would be greatest during spring and summer.

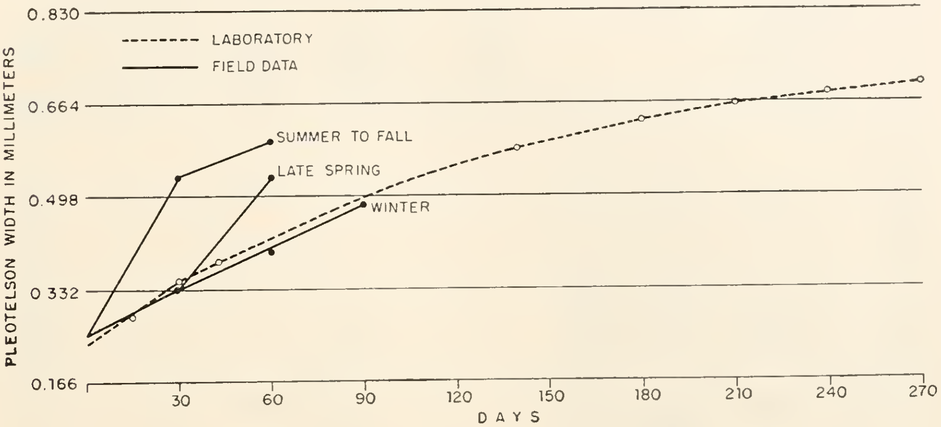

FIGURE 4. *Limnoria tripunctata*. Individual growth rate at different seasons: late spring, average temperature 19° C.; summer to fall, average temperature 22° C.; winter, average temperature 15° C.; laboratory culture average temperature 15° C. Measurements in field are from broods hatched on test blocks, those in laboratory, from individual specimens.

Population Growth.

Some idea can be had of the population growth by direct observation of the number and size of broods produced by migrant females during different portions of the year, namely on test blocks that were in place for successive 2-, 3- and 4-month periods throughout the year. In Table VIII summaries and calculations of probable annual rate of reproduction have been made, based on number of young observed in these 2- to 4-month periods.

Data from the test blocks show that a brood may be produced in a minimum of 25 days. The three- and four-month exposures indicate, however, that only one brood is produced during the winter, possibly three during the summer and at most only two during spring and fall. Thus a maximum of eight broods could conceivably result if a female can continue to produce successive broods at this rate. It has, however, been possible to follow the performance of individual females through a maximum of only three successive broods. Hence, it is not known whether or not a female that has produced three broods during the season of maximum production will continue to produce additional broods at the reduced rate at other seasons. Therefore, extrapolations must be considered with caution. Assuming an annual rate of eight broods with an average of 10 young per brood (Table VI) 100 females might produce a maximum of 8000 young. However, not all females in a population are gravid at any one time. The gravidity may be close to 100% during the mid-summer but drops as low as 4% during the winter (Table VII). An approximate mean gravidity for the year might be near 60% which would allow 100 females to produce only 4800 young per year at the rate shown for test board populations. Females in old piling populations appear to produce only about ½ this figure since their average brood size is only 5.

TABLE VIII

Limnoria tripunctata. Rate of production of young based on observed values for test block populations

Station	Duration of test block exposure	Dates of exposure	Total number of months	Number of young found per 100 mature females	Raw monthly rate	Adjusted* monthly rate	Adjusted* annual rate
A	2 months	Aug. 1952– Oct. 1953	12	380	31.6	63.2	758
A	2 months	Oct. 1953– Dec. 1954	12	717	59.7	119.4	1433
B	2 months	Feb. 1954– Feb. 1955	12	619	51.6	103.2	1238
A	3 months	April 1954– Jan. 1955	9	1009	112.1	149.1	1789
B	3 months	Feb. 1954– Jan. 1955	9	1251	139.0	185.3	2224
B	4 months	Feb. 1954– Feb. 1955	12	1585	132.0	165.0	1980
					Average annual rate		1570

* In order to approach the reproductive continuity that must occur on piling, one-month incubation time is allowed. Raw monthly rate on blocks of 2-month exposure are accordingly doubled, those of 3- and 4-month exposures are increased by ⅓ and ¼ of raw rate, respectively.

If the average annual realized reproductive performance of 100 sexually mature females is 1570 young as shown in Table VIII, the difference between this figure and the reproductive potential based on number and size of brood suggests the annual mortality on test blocks.

The reproductive rate given above may appear low compared with the potentialities suggested for *Limnoria lignorum* by Kramp (1927) who indicated four broods of 20–30 eggs per female per year. He, however, did not investigate the relative number of young resulting on the test blocks. Hence, no allowance was made for mortality. In Japan Shiino (1950) found only 1–12 eggs produced by *L. lignorum* (*tripunctata?*).

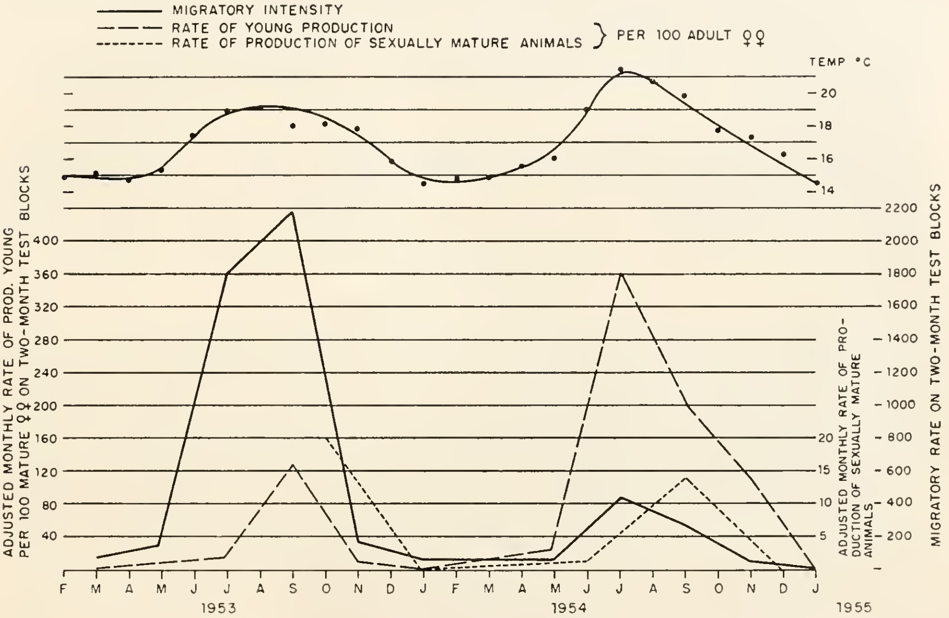

FIGURE 5. *Limnoria tripunctata.* The relationship between monthly production rates and migration in two-month periods, Station A, San Diego Bay.

The relationship between monthly production rate and migration in San Diego Bay is shown graphically in Figure 5. Interpolated to the piling, these data indicate that a marked increase would have occurred there during the months of maximal migration.

SUMMARY

1. *L. tripunctata* undergoes a definite seasonal migratory period which reaches its height approximately during the season of highest water temperatures. This differs from the earlier migratory season usually shown for *L. lignorum*.

2. The attack on new wood is about equally severe on floating and submerged blocks.

3. A comparison of the percentage composition of the 30-day test-block populations shows that about 97% of the migrants are sexually mature animals as con-

trasted with piling populations where 50% consisted of newly hatched immature animals and only about 28% sexually mature. The piling population remains fairly constant in its composition.

4. None of the females is gravid at the time of migration. Pairing takes place after establishment on the test blocks.

5. As with *L. lignorum* the factors influencing migration are probably a combination of approach to a reproductive phase and population pressure due to overcrowding in old established populations.

6. Water temperature is important in influencing migration in that the higher temperatures hasten the rate of growth and attainment of sexual maturity, production of eggs, and shorten the incubation period. Gravidity (per cent gravid females) in *L. tripunctata* evidently reaches its peak between 17° and 19° C. as contrasted with about 10° C. for *L. lignorum*.

7. The brood size of piling populations is about ½ that found in uncrowded test-block populations.

8. A single female probably produced three or more broods during the year.

9. Using size as a criterion, most migrant females appear to have produced a brood prior to migration.

10. In extrapolating the test-block data to the piling populations, there is indicated a marked population increase during the months of maximal migration.

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THE OCCURRENCE OF PORPHYRIN IN THE PLANARIAN[1]

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During a study of electrophoretic properties of protein extracts of planarians, there was observed a substance characterized by a bright red fluorescence under ultra-violet light and a high electrophoretic mobility. The fluorescence suggested the substance could be a porphyrin or a related compound. Recent reviews in comparative animal biochemistry, although treating the occurrence of porphyrins, give no information as to the occurrence of porphyrin in planarians (Baldwin, 1939; Florkin, 1947; Prosser *et al.*, 1950).

An investigation of this substance with regard to some of its chemical properties identifying it as a porphyrin will be reported in this paper, and the possible biological significance will be discussed.

MATERIALS

The biological material used was the planarian, *Dugesia dorotocephala;* the species is distinguished from related species by its large size (20–30 mm.) and dark pigment (Hyman, 1951). The planarians were collected in a pool on the Berkeley campus of the University of California. The water of the pool was covered by a dense growth of water fern among which the planarians were found. The temperature of the water varied between 14° and 16° C. during the summer when the animals were collected and studied. The animals were used for experiments on the next day or the same day as collected, unless stated otherwise.

EXPERIMENTAL DATA

1. Preparation of extracts and preliminary results

The planarians were washed with distilled water and then homogenized in a Potter-type homogenizer. Distilled water was added to wash out the thick suspension. This water extract was centrifuged at low speed (about 500 rpm). The supernatant was discarded; the dark residue was resuspended in distilled water and centrifuged again. The washed residue was suspended in 0.01 *M* sodium carbonate at pH 11.0, in which solution almost all the residue dissolves. This was centrifuged and the pale brownish supernatant contained the substance fluorescing red under ultra-violet light. The original supernatant of the water extract was examined under ultra-violet; this possesses no red fluorescence but rather a pale gray fluorescence. No red fluorescence could be produced in the water-extracted supernatant by adding sodium carbonate up to the pH at which the dissolved residue

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[1] This investigation was supported by a research grant (C-2410 M and G) from the National Cancer Institute, of the National Institutes of Health, Public Health Service.

fluoresces. This suggested that there was a red fluorescent substance bound to or adsorbed on the sedimentable granules that was not eluted with water but was extracted or eluted with aqueous sodium carbonate at pH 11.0.

The absorption spectrum of the residue dissolved in Na_2CO_3 was ascertained using a Beckman spectrophotometer. The spectrum showed a sharp absorption band near 400 mμ, as well as several weaker bands at the longer wave-lengths. The sharp absorption near 400 mμ is characteristic of the porphyrin ring and is found for all porphyrins regardless of their side chains. This band is termed the Soret band. The spectrum of the substance in Na_2CO_3 served only for its identification as a porphyrin. Using HCl as a solvent revealed the spectrum more clearly and permitted comparison with absorption maxima of porphyrins as reported in the literature; these maxima will be presented later in the paper.

2. Solubility study

The solubility of the porphyrin in various solvents was determined by suspending the water-insoluble residue in the solvent; the porphyrin was considered soluble if ultra-violet light[2] revealed red fluorescence in the solution. By this criterion, the porphyrin was found to be soluble in Na_2CO_3 (0.01 *M*), KOH (0.1 N), NH_4OH (10%), HCl (5% and 25%), and ethyl acetate (in acetic acid); it was insoluble in ethyl alcohol (95%), acetic acid (1%), chloroform, acetic-ether and ether. The fact that the porphyrin is insoluble in ether places it in the category of ether-insoluble porphyrins. This eliminates the possibility that the porphyrin in question is a protoporphyrin, mesoporphyrin, deuteroporphyrin or coproporphyrin, all of which are ether-soluble. The insolubility in ether and its solubility in ethyl acetate (in acetic acid) are characteristic of uroporphyrins, which are porphyrin octacarboxylic acids unextractable in ether or chloroform unless the carboxylic acid groups are esterified (Lemberg and Legge, 1949).

The methyl ester of the porphyrin was prepared by adding an acid solution of the porphyrin to cold methyl alcohol saturated with HCl, and allowing the solution to remain at 5° C. for several days. The porphyrin ester can then be extracted with chloroform. A hydrolysis of the ester can be achieved by adding 25% HCl to the methyl ester in chloroform and allowing the mixture to stand at 5° C. for several days. The red fluorescence of the porphyrin then appears in the top aqueous acid layer. This procedure was used for purification of the porphyrin for absorption spectrum studies.

3. Absorption spectrum study

The absorption spectrum of the planarian porphyrin in 5% HCl revealed maxima at I 600 mμ, II 555 mμ, III 495 mμ, and IV 406 mμ; order of intensity IV, III, II, I. When the porphyrin was dissolved in 25% HCl the maxima were I 598 mμ, II 553 mμ, III 496 mμ, and IV 409 mμ (see Fig. 1). The maxima reported by Grinstein, Schwartz and Watson (1945) for purified uroporphyrins in 25% HCl are: uroporphyrin (204° C. melting point) I 597 mμ, II 553 mμ, III 410 mμ; uroporphyrin (284° C. melting point) I 595 mμ, II 552 mμ, III 408 mμ.

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[2] The source of ultra-violet radiation was a 6 watt GE360 Black Light lamp which produces long wave (3660 Å) ultra-violet. The lamp was supplied by Vogel Luminescence Corp.

The absorption spectrum of the methyl ester of the porphyrin in chloroform had maxima at I 626 mμ, II 582 mμ, III 575 mμ, IV 535 mμ, V 502 mμ, VI 408 mμ. The maxima reported by Grinstein *et al.* (1945) for the methyl ester of uroporphyrin in chloroform are I 626 mμ, II 581.5 mμ, III 570.5 mμ, IV 536 mμ, V 501.5 mμ, VI 408 mμ.

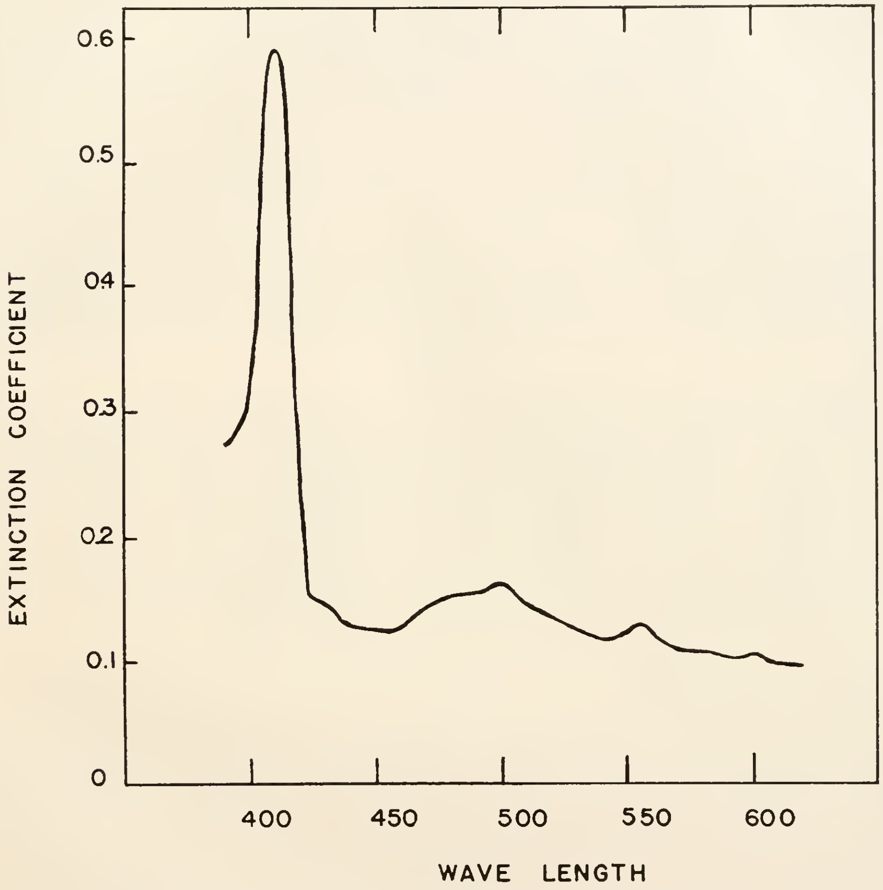

FIGURE 1. The absorption spectrum of the planarian porphyrin extracted in 25% HCl directly from water-insoluble granules is shown. The extinction coefficient ($\log \frac{I_o}{I}$) is plotted against the wave-length (in millimicrons).

After hydrolysis of the methyl ester with 25% HCl, the purified planarian porphyrin had maxima at I 596 mμ, II 553 mμ, (511 mμ) III 409 mμ; order of intensity III, II, I. After purification by esterification and hydrolysis, the absorption at 496 mμ is removed; this suggests that the absorption at 496 mμ was due to another substance, perhaps a related compound.

There is considerable similarity between the absorption spectrum of the porphyrin from planarians and the uroporphyrin isolated by Grinstein *et al.* (1945) from urine of patients with a congenital porphyria which is characterized by excretion of large quantities of uroporphyrin.

4. Paper electrophoresis

The mobility of the porphyrin was observed with paper electrophoresis using a somewhat modified technique of Kunkel and Tiselius (1951). Whatman 3MM filter paper was held between glass plates and dipped at the ends into electrode vessels containing Na_2CO_3 (0.01 M), $\mu = 0.03$, pH 11.0. The porphyrin solution was placed on the paper at the cathode end 17 cms. from the cathode and 40 cms. from the anode. The run was made at room temperature using 200 volts and 3–5 milliamperes. Serum albumin stained with brom phenol blue was applied parallel to the porphyrin for comparison of relative mobilities. Both the stained albumin and the red fluorescent spot of the porphyrin moved as discrete areas towards the anode. The location of the anterior edge of the two moving areas was noted at intervals. The distance moved for porphyrin and albumin, respectively, was 5.7 cms. and 3.8 cms. after one hour; 9.7 cms. and 6.0 cms. after two hours. The ratios of their relative mobilities were 1.45 at one hour and 1.6 at two hours.

To locate protein at the completion of the run, the paper was immersed in 10% $HgCl_2$ in 95% ethyl alcohol, stained in brom phenol blue (0.1% in 95% ethyl alcohol) for one hour, destained in 1% acetic acid. No protein stain was observed at the final location of the red fluorescence, although the protein staining revealed a protein of low mobility not associated with the red fluorescence under these conditions of electrophoresis. The protein may have dissociated itself from the porphyrin during the electrophoresis or may be a separate protein found in the residue and extracted by the same alkaline solvent as the porphyrin.

5. Paper chromatography

The identification of the planarian porphyrin was checked by paper partition chromatography using a lutidine-water system described by Nicholas and Rimington (1951). There is a linear relationship between R_F values and the number of carboxyl groups in the porphyrins and this can be used to identify the porphyrin.

The porphyrin was extracted in 2 N NH_4OH or 1% HCl and applied in the solvents to pieces (12 × 15 cms.) of Whatman No. 1 filter paper along a base line 2 cm. from the lower edge. The chromatographs were run in the dark at 21° C. in a lutidine-water system containing 40% water by volume. The position of the spots after development was determined by their fluorescence in ultra-violet light.

The porphyrins run were the planarian porphyrin assumed to be a uroporphyrin and a known sample of hematoporphyrin. R_F values were calculated and compared. Under these experimental conditions, the R_F value of the hematoporphyrin was between 0.7 and 0.8; the R_F value of the planarian porphyrin was between 0.01 and 0.1. The R_F values reported by Nicholas and Rimington (1951) for two-carboxyl porphyrins (such as hematoporphyrin) were about 0.8 and for eight-carboxyl porphyrins (uroporphyrin) were about 0.1 under experimental conditions similar to those used in these experiments. This suggests that the planarian porphyrin contains eight carboxyl groups and may be uroporphyrin.[3]

6. Content of porphyrin in planarians

To determine whether porphyrin content varied from one planarian to another, different numbers of animals of approximately the same size were collected and the relative amount of porphyrin determined. The extinction coefficient ($\log \frac{I_o}{I}$) at the maximum absorption, 408 mμ, was used as the measure of porphyrin content. Since planarians vary in size, it is necessary to relate the porphyrin to some unit of planarian size or weight. Wet weight was chosen as the most convenient measure. Since the porphyrin is insoluble in ethyl alcohol, this can be used as a means of killing or fixing the animals and thus facilitating wet weight determinations. Before extraction of the porphyrin, a known number of planarians was placed in a tared watch glass, the animals were immediately killed with 95% ethyl alcohol, and filter paper was applied to remove excess moisture before weighing. After weighing, the planarians were homogenized in water, centrifuged, the residue extracted in a constant volume (3 ml.) of 25% HCl, and the extinction coefficient determined. The porphyrin coefficient was stated in arbitrary units as the extinction at 408 mμ per total wet weight. The data are presented in Table I. Under similar physiological conditions, the unit of porphyrin per planarian of a given wet weight may be a constant.

TABLE I

Number of planarians	Extinction at 408 mμ	Total wet weight (mg.)	Ratio (E/weight)
2	0.081	13.7	0.0059
4	0.256	41.6	0.0061
6	0.335	51.7	0.0064
8	0.412	60.9	0.0067
10	0.482	71.2	0.0067

Preliminary experiments indicate that the porphyrin is also present in animals which had been starved in the laboratory for one, two and four weeks. Porphyrin is present in regenerating and regenerated animals. The porphyrin is not confined to any one region of the animal; it is distributed throughout the animal although the distribution may not be uniform along the length of the animal.

7. Visual demonstration of porphyrin

A visual demonstration of porphyrin within the planarian can be made by exposing animals to ultra-violet light after they have been immersed in 95% ethyl alcohol. One can then observe the whole planarian emitting a red fluorescence. No definite fluorescence could be detected in the living animal, although the water-insoluble sedimentable granules do show some fluorescence without further chemical treatment. The alcohol removes from the animal a substance or substances which may obscure the red fluorescence. This substance is yellow in solution, has a yellow fluorescence, and is soluble in organic solvents such as ethyl alcohol, methyl alcohol, acetone, chloroform and pyridine. Any of these organic solvents can remove the yellow substance from the whole animal and allow the red fluorescence of the porphyrin to be observed. This yellow substance is probably a lipochrome or carotenoid, and requires further investigation.

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[3] A sample of the planarian porphyrin was analyzed recently in the laboratory of Dr. Samuel Schwartz of the Department of Medicine at the University of Minnesota. More refined techniques of chromatography on calcium carbonate revealed two red fluorescent zones. Paper chromatography showed the presence of coproporphyrin and a porphyrin which behaves like uroporphyrin. The author wishes to thank Dr. Samuel Schwartz, as well as Dr. Marie Berg and Mr. Micheal Keprios for their analysis of this material.

Discussion

Uroporphyrins were first described by Fischer in 1915 and since then much work has been done to characterize and describe this substance (see review by Lemberg and Legge, 1949). The main source of uroporphyrin has been the urine of patients having congenital porphyria (Waldenström *et al.*, 1935; Grinstein *et al.*, 1945). In the case of porphyria the occurrence of uroporphyrin is considered to be a metabolic error. However, in the case of the planarian, this porphyrin seems to be found in all individuals and may occur as a physiological porphyria, as Turner (1937) described the case in the fox-squirrel where uroporphyrin occurs normally in the bones. Uroporphyrin is found normally in small amounts in the zones of calcification of foetal bones and it is found in larger amounts in the bones and teeth of congenital porphyria cases in man and other mammals (Rimington, 1955). Uroporphyrin occurs in the *Pteria* mussel shell (Fischer and Haarer, 1932; Nicholas and Comfort, 1949) and occurs as a copper complex in the flight feathers of the African turacos (Fischer and Hilger, 1924). In these cases the uroporphyrin seems to be stored in metabolically rather inert tissues, except perhaps for foetal bones. This suggests that the uroporphyrin may be an end-product of chemical events, perhaps of calcification in some cases. Uroporphyrin has been postulated to be in the biosynthetic pathway leading to the formation of haem, a porphyrin containing substance vital to higher organisms, but recent studies suggest that uroporphyrin may be a side reaction and not involved in the biosynthetic pathway to haem (see review of Rimington, 1955). Thus uroporphyrin may be an end product whenever it occurs, either in pathological urine, bones, teeth, feathers, shells or in planarians. However, in the case of the planarian, the porphyrin is distributed rather generally and is not stored in inert tissue. This raises the question whether there may not be some biological significance for the planarian porphyrin.

Loeb (1893) and Hesse (1897) had independently observed that the movements of planarians were influenced by light. Parker and Burnett (1900) extended these observations and measured the speed of movement of planarians away from light; they found that animals with and without eyes responded so as to move away from light but the eyeless ones at a somewhat slower rate than those with eyes. It has also been shown that planarians perceive ultra-violet radiations and produce a negative reaction; the behavior is the same for normal and eyeless specimens (Werner, 1926). Short exposures to ultra-violet and to direct sunlight are fatal to planarians (Merker and Gilbert, 1932).

The question is whether this sensitivity and response to ultra-violet may wholly or in part perhaps be due to the presence of porphyrins which are strongly photoactive and efficient in absorbing light around 4000 Å. This response may be related to the photosensitizing action of porphyrins discovered in 1909 by Hausmann (as reported by Lemberg and Legge, 1949). He found that experimental animals and humans injected with porphyrin solutions and then exposed to sunlight or ultraviolet light, showed a skin sensitization. Also it is known that human individuals suffering from congenital porphyria show a marked light sensitization of the skin (Lemberg and Legge, 1949). In planarians the light sensitization may have a normal function to produce the avoidance of light.

The author wishes to acknowledge the technical assistance of Miss Emily Martin.

SUMMARY

1. A porphyrin was extracted from the water-insoluble residue of homogenates of the planarian, *Dugesia dorotocephala*. The porphyrin extract is characterized by a red fluorescence under ultra-violet light.

2. The solubility properties of the porphyrin, the absorption spectra of the porphyrin and its methyl ester, and paper chromatographic behavior show a similarity to those described for uroporphyrin.

3. The amount of porphyrin seems to be a constant per planarian of a given wet weight, when the animals are under the same physiological condition. The porphyrin is also found in starved and in regenerating animals.

4. The whole planarian emits a red fluorescence under ultra-violet light if the animal is first immersed in one of several organic solvents which remove a lipochrome or carotenoid substance.

5. The light sensitivity which has often been described for these animals may to some degree be due to the presence of the porphyrin.

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OXYGEN CONSUMPTION OF WHOLE INSECTS AND INSECT HOMOGENATES[1]

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The alteration of enzyme activity by cell rupture has been extensively studied with vertebrate tissues, but very little work has been done with those of invertebrates. Bodine and Lu (1951) found that homogenization of both diapause and actively growing embryos of the grasshopper, *Melanoplus differentialis,* reduced oxygen consumption by approximately 65 per cent. Their results agree with the 60 to 70 per cent decrease obtained with vertebrate tissue (De Robertis, Nowinski and Saez, 1950). They concluded that 65 per cent of the oxygen uptake of intact embryos is due to structure and the remainder to the basic chemical composition of the cells.

In the present study, the oxygen consumption of homogenates of the Japanese beetle, *Popillia japonica,* and of the mealworm, *Tenebrio molitor,* was determined for each day of embryonic development at 30° C. A comparison was then made with the oxygen consumption of whole eggs. They lack a diapause and thus differ from the eggs of the grasshopper, *M. differentialis.* A similar comparison was made with whole insects and their homogenates in the larval stage, and for each day of the pupal stage of the mealworm at 30° C. Finally, a study was made of the effect of different homogenate concentrations on the oxygen consumption of larvae and pupae of the mealworm.

MATERIAL AND METHODS

Japanese beetle eggs were collected daily and placed on the surface of moist, firmly-packed soil in one-ounce metal salve boxes. Eggs of the mealworm were collected daily from beetles kept in white flour. They were stored in a desiccator over a saturated solution of NaCl (relative humidity, 75 per cent). Eggs of both species were incubated at 30° C.

Larvae of the mealworm, obtained from a mixed culture maintained at room temperature, were used in these experiments. Prepupae were collected from the same culture and placed at 30° C. They were examined daily and the newly molted pupae were placed in beakers and kept at 30° C. In this manner, pupae of known ages (within 24 hours) were obtained.

All homogenates were made in 0.03 molar phosphate buffer, adjusted to a pH of 7.4. The tissue was homogenized for one minute in a motor-driven glass homogenizer. This time was selected because no differences were found in the respiratory rates of tissues homogenized for one, three, or five minutes.

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Oxygen consumption was measured in Warburg constant volume manometers according to the procedure outlined by Umbreit, Burris and Stauffer (1945). The center well of the Warburg flask contained 0.2 ml. of a 10 per cent KOH solution to which was added a piece of folded filter paper to increase the surface of the KOH

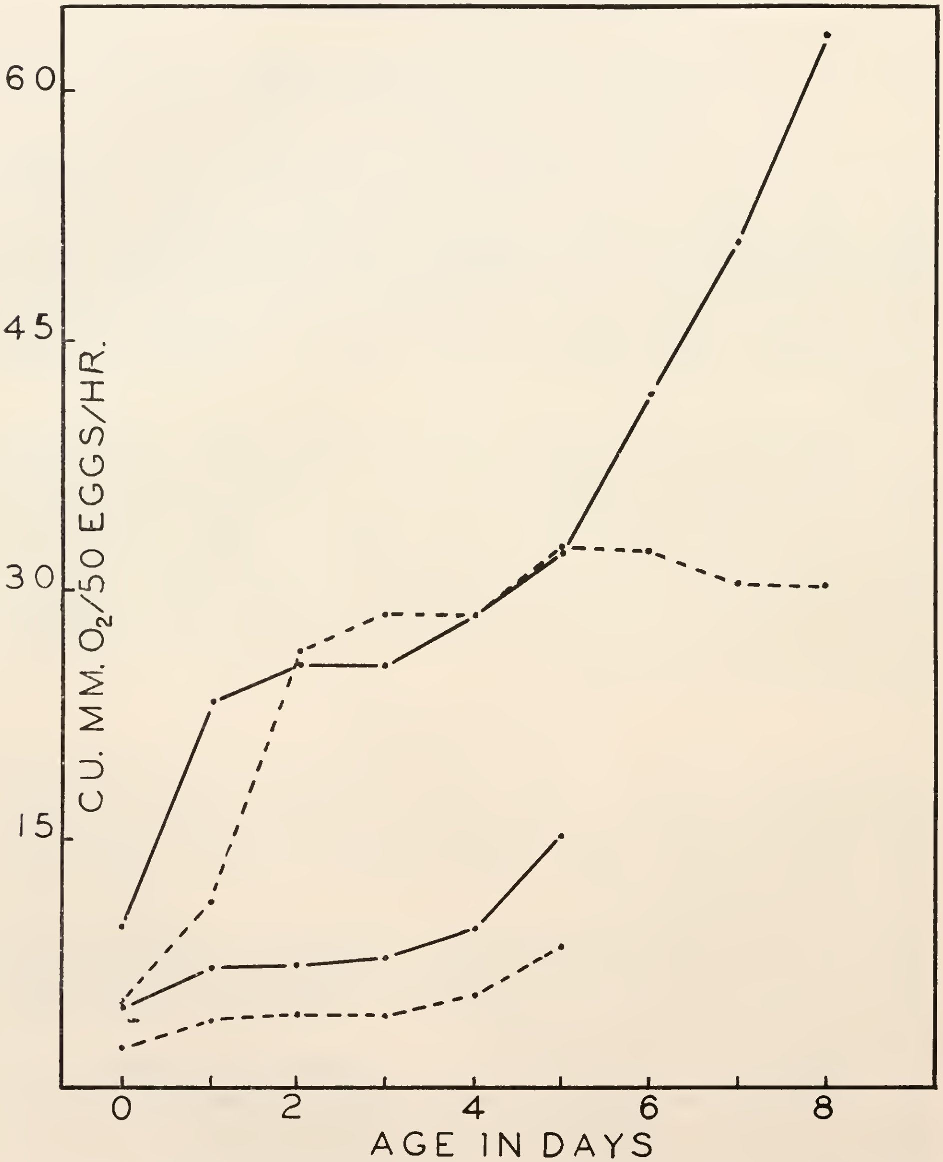

FIGURE 1. Effect of homogenization on the O_2 consumption of insect eggs. Upper graphs, Japanese beetle eggs; lower graphs, mealworm eggs; solid lines, intact eggs; dotted lines, 10 per cent homogenates. Graph for intact Japanese beetle eggs taken from Ludwig and Wugmeister (1955).

TABLE I

Oxygen consumption of whole mealworm larvae and of homogenates of mealworm larvae

Per cent homogenate	No. of readings	Oxygen consumption μ liters/gram/minute (with standard errors)	Per cent decrease
Whole larvae	46	10.17 ± 0.73	
20	18	7.25 ± 0.28	28.7
10	71	6.62 ± 0.34	34.9
7.5	18	7.45 ± 0.73	26.7
5	42	6.06 ± 0.33	40.4
2.5	18	5.46 ± 0.58	46.3
1	49	3.65 ± 0.34	64.1

solution. The manometers were placed at a temperature of 30° ± 0.01° C. Readings were made at 15-minute intervals for a period of two hours. During this time the manometers were rocked horizontally 100 times per minute.

OBSERVATIONS

A comparison of the rates of oxygen consumption of whole Japanese beetle eggs and of their homogenates for each day of embryonic development is shown in Figure 1. Each point in this figure represents an average of at least 10 determinations. Respiration of 10 per cent homogenates, expressed as μ liters of oxygen/50 eggs/hour, was 5.1 in newly laid eggs. It increased to 26.2 during the next two days and remained relatively constant at approximately 30 during the remainder of the embryonic period. A comparison of these results with those of Ludwig and Wugmeister (1955) shows that homogenizing the newly laid egg reduced oxygen consumption by approximately 50 per cent. It did not change the respiratory rate from the third to the sixth days of incubation, but towards the end of the embryonic period, it again resulted in a reduction of more than 50 per cent. On the other hand, the amount of reduction was maintained constant throughout the embryonic period in the mealworm. Using 10 per cent homogenates, it was found that homogenizing the egg of this species resulted in a reduction of approximately 48 per cent in the rate of oxygen consumption (Fig. 1).

The rates of oxygen consumption of whole mealworm larvae and of their homogenates are listed in Table I. Oxygen consumption of whole larvae averaged 10.17 μ liters/gram/minute. That of homogenates depended upon concentration, being 7.25 in 20 per cent, and only 3.65 in 1 per cent homogenates. In the former case, the reduction amounted to approximately 29 per cent; and in the latter, to 64 per cent.

The effects of homogenization on the rate of oxygen consumption of the mealworm during metamorphosis are shown in Figure 2. Each point in Graph A represents an average of at least 10, in Graph B of 30, and in Graph C of 12 determinations. The oxygen consumption of whole insects followed the characteristic U-shaped curve first described for this species by Krogh (1914). It was high immediately after pupation, decreased to a minimum in the two-day pupa, and then increased during the remainder of the pupal stage. The oxygen uptake of 1 per cent and of 5 per cent homogenates remained relatively constant during the pupal stage, but increased in newly emerged adults. Hence, the amount of reduction resulting from homogenization varied with the stage of metamorphosis. With 1 per cent homogenates, it was approximately 61 per cent in prepupae, 80 per cent in newly molted, 50 per cent in two-day, 72 per cent in five-day pupae, and 80 per cent in newly emerged adults. The oxygen uptake of 5 per cent homogenates was considerably higher than that of the 1 per cent. However, its rate was also relatively constant throughout the pupal stage.

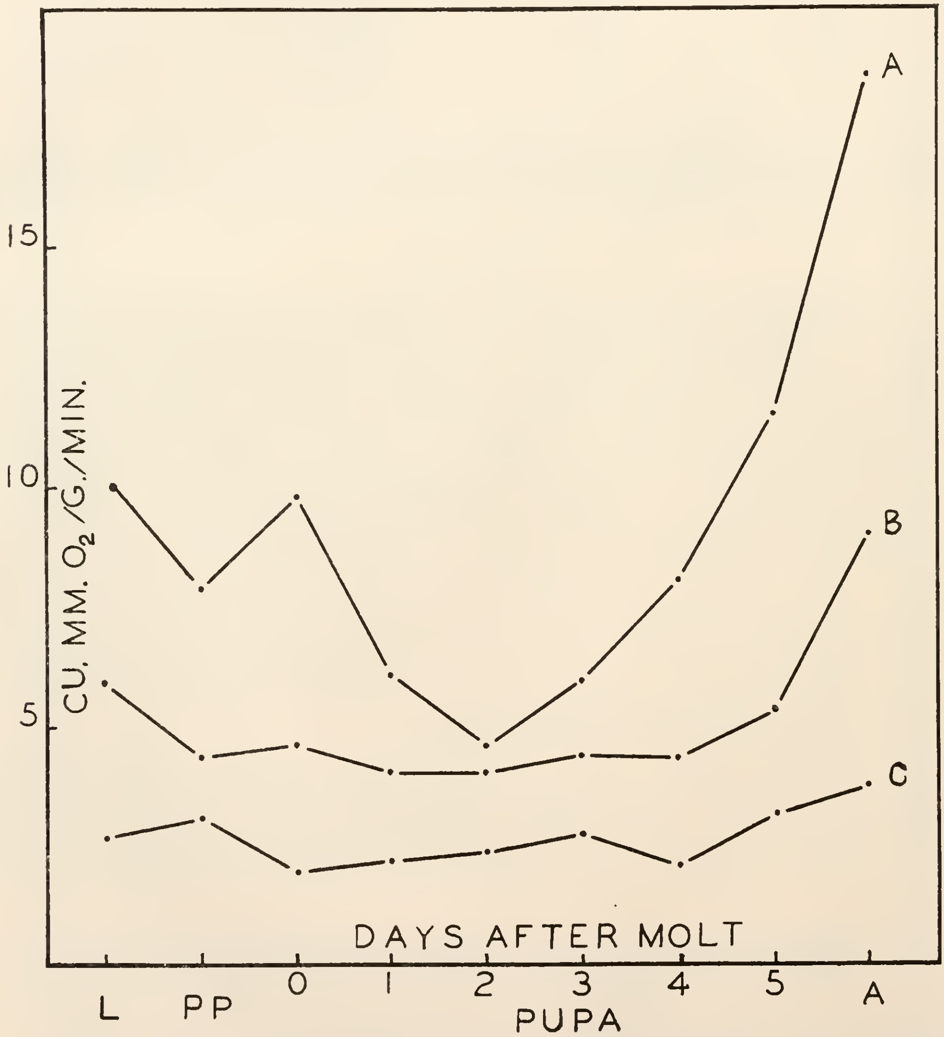

FIGURE 2. Effect of homogenization on the O_2 consumption of mealworms during metamorphosis. Graph A, intact insects; graph B, 5 per cent homogenates; graph C, 1 per cent homogenates; L, larvae; PP, prepupae; A, newly emerged adults.

DISCUSSION

The varying effects of homogenization on oxygen consumption throughout the embryonic period of the Japanese beetle may possibly be correlated with water content and degree of organization of the egg. Rothstein (1952) reported that the newly laid egg weighed only 0.83 mg. and contained 50 per cent water. Hence, the various constituents are in a rather concentrated condition. During the first four days of development at 30° C., the egg increased in weight to 2.3 mg. and its water content increased to more than 80 per cent. Hence, approximately 1.5 mg. of water were imbibed by each egg and the enzymes and other constituents were greatly diluted. At this time homogenization appears to have no effect on the rate of oxygen consumption. Gese (1953) found that blastokinesis occurs on the fifth day at 30° C., followed by the differentiation of the embryo. This period of differentiation was shown by Ludwig and Wugmeister (1955) to be associated with a rapid increase in respiratory rate and an increase in the activities of cytochrome oxidase and succinic dehydrogenase. Homogenization of the egg during this period of differentiation resulted in a marked reduction in oxygen consumption, probably due to the disruption of organization in the egg. On the other hand, mealworm eggs do not imbibe water but maintain a constant weight throughout the embryonic period. Homogenization of these eggs resulted in a constant reduction of approximately 48 per cent in oxygen consumption. The difference between the present results with mealworm eggs, and those of Bodine and Lu (1951) with grasshopper eggs (48 as compared to 65 per cent), may be associated with homogenate concentration. Furthermore, Bodine and Lu used embryos only, while in the present experiments whole eggs were used.

The elimination of the U-shaped respiratory curve in the pupal stage of an insect by homogenization was also reported by Cotty (1955) with the house fly, *Musca domestica*. These observations add evidence for the belief that the U-shaped curve is associated with different degrees of tissue organization (Krogh, 1914; Fink, 1925). When this organization is disrupted by homogenization, a uniform rate is produced. However, homogenization may also affect respiratory enzymes and thus modify the rate of oxygen consumption as indicated by the work of Handler and Klein (1942) who showed that DPN is rapidly broken down by tissue homogenates thus blocking various dehydrogenases.

The present experiments indicate that the effects of homogenization on the respiratory rate of insect tissues vary with the stage of development, physiological differences among various species, and homogenate concentration.

SUMMARY

1. The oxygen consumption of 10 per cent homogenates of Japanese beetle and mealworm eggs was measured for each day of the embryonic period at 30° C. and compared with that of intact eggs.

2. Homogenization of newly laid Japanese beetle eggs resulted in a reduction of 50 per cent in oxygen consumption. From the third to sixth day, there was no decrease but towards the end of the embryonic period, a reduction of more than 50 per cent was again obtained. Homogenization of mealworm eggs resulted in an average reduction of 48 per cent in oxygen consumption which remained constant throughout the embryonic period.

3. The rates of oxygen consumption of whole mealworm larvae and of their homogenates were determined. Homogenization resulted in a decrease in respiratory rate, the extent depending on homogenate concentration. With 20 per cent homogenates, there was a reduction of 29 per cent; and with 1 per cent homogenates, it was 64 per cent.

4. The effects of homogenization on the respiratory rate of mealworms were determined for each day of metamorphosis at 30° C. Homogenization abolished the U-shaped respiratory curve characteristic of intact pupae. Therefore, the degree of reduction depended on the stage of metamorphosis. With 1 per cent homogenates, it was 61 per cent in prepupae, 80 per cent in newly molted, 50 per cent in two-day, 72 per cent in five-day pupae, and 80 per cent in newly emerged adults. Furthermore, the extent of reduction also varied with homogenate concentration being greater in 1 per cent than in 5 per cent homogenates.

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RECIPROCAL PROTOZOAN TRANSFAUNATIONS BETWEEN THE ROACH, CRYPTOCERCUS, AND THE TERMITE, ZOOTERMOPSIS[1]

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A technique has recently been described whereby the symbiotic protozoa of the wood-feeding roach, *Cryptocercus,* may be transferred from the hind-gut of one host to another *per rectum* (Cleveland and Nutting, *J. Exp. Zool.,* in press). The method has now been applied successfully in the reciprocal transfer of protozoa between this roach and the large, western North American termite, *Zootermopsis.*

An earlier series of experiments by Cleveland, in which the transfer was accomplished by feeding, showed that the protozoa were capable of living in alternate hosts for some time (Cleveland *et al.,* 1934, p. 320). This preliminary work called for more extensive experiments, first to determine whether the termite protozoa could indeed supplant the normal infection of the roach and vice versa. Should this then be the case, it would be a matter of considerable interest to determine the behavior of the protozoa in their new hosts during the molting period.

Experimentation of this sort has been extremely limited and largely confined to determining the host specificity of protozoa from certain termites. Light and Sanford (1928) reinfected oxygen-defaunated termites *per rectum* with a glass canula connected by rubber tubing to a mouthpiece. They found that the protozoa of *Zootermopsis angusticollis* could live and multiply in *Kalotermes hubbardi* for at least 100 days. Dropkin (1941) similarly infected *Z. angusticollis* with protozoa from *Reticulitermes flavipes,* and *Kalotermes jouteli* and *schwarzi.* None of the protozoa multiplied, and so were unable to support the new host for longer than 50 days. He therefore concluded that these unsuccessful refaunations resulted from physiological as well as morphological distinctions which must exist between the protozoa from widely different species of termites.

Before describing the roach-termite transfaunations, it is pertinent to recount the natural methods for transfer of protozoa between the normal hosts. *Cryptocercus* harbors a large and diverse fauna composed of 13 described genera and some 30 species of hypermastigote and polymastigote flagellates. All of these genera change from asexual to sexual methods of reproduction during the molting period of their host which, in the laboratory at 21° C., lasts for about 44 days. Most of the individuals of *Trichonympha* encyst a few days before molting, and large numbers are usually passed in the fecal pellets within 20 to 36 hours after ecdysis. The hatching of nymphs from their oothecae coincides with the peak of the molting season, so that the new individuals are able to gain their initial infections, at least in

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part, by feeding on these pellets. This infection is never lost naturally, even during succeeding ecdyses. Adults and intermolt nymphs do not normally pass protozoa in their fecal pellets, except for an occasional small polymastigote (Cleveland *et al.*, 1934). From observations made on incipient colonies in nature, it is becoming evident that there must be some other means of transferring these protozoa from intermolt nymphs, and probably adults, to the newly hatched nymphs. Repeated attempts to discover such a method in the laboratory have thus far been unsuccessful.

On the other hand, the five genera and seven species of flagellates symbiotic in the hind-gut of *Zootermopsis* do not undergo any sexual cycles, and die shortly before each ecdysis. Occasionally, a few individuals of *Tricercomitus* and/or *Streblomastix* remain in the hind-gut through this period. These recently molted and defaunated termites regain their infections by the solicitation of proctodaeal "food," which contains active protozoa, from their non-molting associates.

MATERIALS AND METHODS

Specimens of *Cryptocercus punctulatus* Scudder have been collected annually in the vicinity of the Mountain Lake Biological Station of the University of Virginia and maintained in Cambridge for a number of years. Cultures of *Zootermopsis angusticollis* (Hagen) were collected in the vicinity of Placerville, Eldorado County, California, and additional lots from Pacific Grove were kindly supplied by Miss Frances M. Weesner of the University of California, Berkeley. Groups of experimental animals were maintained at 20° C. in petri dishes, supplied with wood and damp paper toweling, and cleaned frequently. This is perhaps not the best method for keeping termites in the laboratory, but it has proven very satisfactory where frequent examination is of first importance.

In brief, the transfaunation technique involves the transfer, *per rectum,* of a fresh inoculum of protozoa to a defaunated recipient. The recipients, either roaches or termites, are previously defaunated under 45 lb. of O_2 for 1.5 hours (Cleveland, 1925a), and later relaxed under CO_2 for the actual transfer. Just prior to the operation the hind-gut contents of the roaches are squeezed out by gentle manipulation of the abdomen. While struggling under CO_2, termites conveniently expel their hind-gut contents almost completely. The inoculum, obtained from the hind-gut of one or more freshly killed donors, is drawn up into a length of polyethylene tubing attached to a syringe. The tube is then guided between the paraprocts into the rectum where the inoculum is released. It is thus possible to replace the original population of a termite with an equivalent volume of protozoa from a roach and vice versa.

EXPERIMENTAL

1. *Transfer of termite protozoa to roaches*

The natural fauna of nine adult and nine nymphal *Cryptocercus* was replaced with protozoa from *Zootermopsis.* In order to examine the entire gut contents and determine the progress of the infection, three adults were killed between 8 and 24 days. In the first, *Trichomonas termopsidis* and *Trichonympha sphaerica* were much more abundant in the anterior than in the posterior colon. The second contained only *Trichomonas,* but in fair numbers. The third contained all forms in normal numbers. Three more were killed for examination after 72 days. These harbored normal numbers of protozoa, but the populations were composed of more *Trichomonas* and fewer *Trichonympha* than are usually found in the termite host.

The remaining adults were examined shortly before presumably normal deaths after 192, 207, and 245 days, for these animals had then been kept in the laboratory for about one year. The first two contained vigorous infections composed of all forms in their usual proportions. Only *Trichomonas* and *Tricercomitus termopsidis* remained in fair abundance in the last adult. It seems probable that other forms were previously present, but died because of the moribund condition of the host.

Of the nine transfaunated *Cryptocercus* nymphs, inoculated with *Zootermopsis* protozoa, three were killed at or near death after 49, 68, and 81 days. One contained a few *Trichomonas* only; the second a few *Trichomonas* and *Trichonympha;* and the third was completely defaunated. Three more were killed after 61, 78, and 83 days, and all had excellent infections. *Trichomonas* was absent from one of these animals, although it is normally very abundant. A sample of protozoa was squeezed from the hind-gut of another nymph after 221 days. All genera and species were present and as abundant and active as though they had just been taken from their original host. Unfortunately, this animal escaped a few days later.

Of the remaining two nymphs, one was examined after 97 days and about one day before ecdysis. *Tricercomitus* and *Streblomastix* were active and present in normal numbers. A few individuals each of *Trichomonas* and *Trichonympha* were behaving normally, but the remainder were either non-motile or already degenerating. The other was examined after 82 days and about 12 hours before ecdysis. *Tricercomitus* was again present in normal numbers, and one *Streblomastix* was seen. No other protozoa were present, but a few unidentifiable remains of larger forms still persisted.

2. *Transfer of roach protozoa to termites*

Fifty-five individuals of *Zootermopsis angusticollis* were defaunated and reinfected with *Cryptocercus* protozoa. These included approximately equal numbers of first, second, and third form reproductives, averaging about 1 cm. long and at least two instars from maturity. Twelve individuals were killed to check the progress of the new infections during the first 50 days. Seven of these had become rather feeble and contained small populations of *Hexamita cryptocerci* and one or two other small polymastigotes, or else were completely defaunated. The remainder contained vigorous, normally proportioned infections. These incomplete faunas, both in transfaunated roaches and termites, are almost certainly due to inadequate refaunation. The early deaths probably resulted from starvation following these poor infections, or from gut injuries incurred during the transfer. Over a period of about one year some 20% of the animals have died in dry or moldy cultures, or have been eaten by other members of the colony for one reason or another. This is not a high mortality figure for these insects maintained in small numbers in such loosely covered containers.

Eight more were examined at ecdysis and at intervals up to 24 hours later.

These were completely defaunated or nearly so. Three individuals contained dead and dying *Trichonympha, Barbulanympha,* and *Saccinobaculus,* along with abundant remains of other large *Cryptocercus* protozoa. A few of them contained insignificant numbers of one or more of the following: *Hexamita* and other very small polymastigotes (from *Cryptocercus*); *Tricercomitus, Trichomonas,* and *Streblomastix* (from *Zootermopsis*). An occasional individual has since been found still harboring a small number of the last three genera, showing that they were not always completely removed by the defaunation time employed here. The three species of *Trichonympha,* dominant forms in the termite, have never been found to survive this treatment.

It should be stated parenthetically that *Streblomastix* is not a symbiont, and is dependent either upon its host or, more probably, upon the other protozoa for its nourishment. *Trichomonas* is symbiotic, but it is doubtful that it alone could maintain its host indefinitely (Cleveland, 1925b). Although the relationships between *Tricercomitus* and *Hexamita* and their hosts have not been studied, it is questionable whether they are truly symbiotic, and if so, whether they could furnish adequate support indefinitely.

Six termites were judged to be approaching ecdysis by reason of their distended appearance and internally divided third and fourth antennal joints. Periodic samples of protozoa were squeezed from these insects and definite evidence of sexual cycles was found in several genera. Several stages of fertilization in *Saccinobaculus* were most commonly seen. These stages ranged from forms where gametes had recently fused, to those in which axostylar fusion was completed and the pronuclei had become juxtaposed (Cleveland, 1950b). Stages of fertilization in *Oxymonas* and *Notila* were also seen in one termite (Cleveland, 1950a, 1950c). *Barbulanympha,* with two nuclei and two flagellated areas (probably gametogenesis), was observed in another (Cleveland, 1953).

In several termites, *Barbulanympha, Trichonympha,* and even the sexually active polymastigotes, were dying or degenerating. One of these termites, which contained *Saccinobaculus* with fusing pronuclei, molted 17 days later and was completely defaunated. The remaining individuals were apparently unable to survive the frequent examinations.

Recently molted termites have repeatedly regained their infections of *Cryptocercus* protozoa from other members of the colony just as they do their own protozoa. Moderate numbers of *Hexamita* and other small polymastigotes become established within 3 or 4 days after molting and adequate refaunations of all protozoa are attained within 15 days, or perhaps less.

Still more convincing evidence in the form of laboratory-established colonies is accumulating to show that *Cryptocercus* protozoa can probably support *Zootermopsis* indefinitely. One third form reproductive (with no indication of wing pads), isolated with five others, began founding an incipient colony in January, 1956. All six of these termites had been infected with roach protozoa seven months previously. Four months later some two dozen nymphs, representing at least three instars, had been produced by this queen. Several of these nymphs were examined and found to contain well balanced faunas of roach protozoa. A second form reproductive (with short wing pads), accompanied by two third form reproductives similarly infected, also began founding a new colony at about the same time.

Within the same period a half-dozen nymphs had been produced. Examination of one of these showed a good infection of *Cryptocercus* protozoa.

3. *Effects of reduced oxygen tension*

The possibility has been raised frequently that the protozoan sexual cycles might be caused by certain changes among the physical factors within the host's hind-gut —most notably a reduction of the oxygen tension. The following experiments, although complete in themselves, more properly form part of a separate and more extensive study of such factors. Their pertinence, however, requires that they be described briefly at this time. Two series of *Zootermopsis* were subjected to varying mixtures of O_2 and N_2 (40% O_2–60% N_2 to 0.5% O_2–99.5% N_2) at atmospheric pressure. In one series the termites contained their own protozoa; in the second, *Cryptocercus* protozoa. It was found that these termites could live in an atmosphere of 2% O_2 and 98% N_2, probably indefinitely. They survived and molted normally under these conditions for about two months. In an atmosphere of 0.5% O_2 and 99.5% N_2 they are nearly defaunated in 24 hours; *Trichomonas* and *Tricercomitus* might survive somewhat longer. The termites themselves were practically immobile during this time, but still recovered when returned to a normal atmosphere. In an atmosphere of 1.0% O_2 the termites survive rather lethargically for two weeks or somewhat longer, but are slowly and irregularly defaunated. Therefore, with the host in the narrow range between 1 and 2% O_2, the protozoa are the first of the partners in the symbiosis to succumb.

Similar experiments, with *Cryptocercus* containing *Zootermopsis* protozoa, have not been carried out although normally faunated roaches are defaunated without injury in two days in an atmosphere of 1% O_2 and 99% N_2. The roaches themselves are unable to recover after three days under these conditions.

The significant point here is that, even under these conditions, the protozoan behavior within the termite is identical to that already observed under normal atmospheric conditions. That is, the termite protozoa continue their usual preecdysial degeneration; not a single organism was seen which could have been considered in the process of sexual reproduction. The roach protozoa continue the initiation of sexual cycles which abort shortly before ecdysis. Reduced oxygen tension by itself, therefore, does not cause the initiation of sexual cycles in either termite or roach protozoa in the termite host. Neither does it assist or hinder the established behavior of the roach protozoa in the termite host. A more thorough discussion on the control of the sexual cycles of the roach protozoa is presented in a separate paper (Cleveland and Nutting, *J. Exp. Zool.*, in press).

Finally, brief mention should be made of the rod-like bacteria (the so-called cuticular striations) regularly found embedded in the pellicle of *Urinympha* and *Barbulanympha*. It is not known whether these microorganisms subserve any nutritive or other function to either protozoa or roach (Cleveland, 1951). They are not normally found on any other protozoan genera in roaches from the Appalachian region, nor on any of the protozoa of *Zootermopsis*. *Urinympha* and *Barbulanympha* retain their embedded bacteria when transferred to the termite, but, what is more interesting, the same or very similar bacteria sometimes become established on the large, protean polymastigote, *Saccinobaculus*, whenever it is so transferred.

DISCUSSION

These experiments demonstrate conclusively that the protozoan complement of *Cryptocercus* can maintain the termite, *Zootermopsis,* through significant portions of two generations and probably indefinitely. They also prove that *Zootermopsis* protozoa can adequately maintain nymphal *Cryptocercus* as long as they do not molt, and mature roaches probably for the normal span of adult life.

The death of the roach protozoa at each molt presents the termite with no more of a problem than does the natural loss of its own protozoa at this time. Aside from the requisite competence of the protozoa themselves, the continuity of this unnatural symbiosis is made possible through the direct method of refaunation, *i.e.,* the solicitation of proctodaeal fluid, already employed by the termites.

On the other hand, the gradual and unnatural loss of termite protozoa during the molting period presents the nymphal roach with an apparently insurmountable problem, and it then dies of starvation. These termite protozoa are as incapable of surviving the molting environment of the roach hind-gut as they are that of their normal host. Earlier experimentation showed that reinfection by the solicitation of proctodaeal food is probably a behavioral impossibility for the roach (Cleveland *et al.,* 1934), although this matter demands further investigation.

Recent work supports the thesis that hormonal mechanisms responsible for growth and molting may also regulate the protozoan sexual cycles in *Cryptocercus* (Cleveland and Nutting, *J. Exp. Zool.,* in press). This work has shown that the synchronization between protozoa and developing host must be very precise in order for the protozoa to accomplish their cycles. The present finding that the roach protozoa actually begin sexual cycles in molting period termites provides encouraging support. The fact that the cycles abort, with resulting degeneration of the protozoa, parallels precisely the behavior of sexual protozoa after they are transferred to another host roach which is more or less advanced in the molting period. It is, therefore, hardly surprising that both roach and termite protozoa are unable to adapt completely to the hormonal milieu of such divergent hosts.

There is much evidence to support the belief that the termites, together with their symbiotic protozoa, are derived from a line of xylophagous roaches. In areas where their ranges are coincident today, *Zootermopsis* and *Cryptocercus* may be found in contiguous galleries within the same log. This fact, together with the present finding that the protozoa of these insects are reciprocally transferable, adds considerable support to the derivation of termites from a line of roaches much like *Cryptocercus* and perhaps even from the same stock.

Finally, a most intriguing observation deserves mention at this time, for it is strongly suggestive of a radical change in the hormonal milieu of the termite host at the time of the imaginal molt. Last stage reproductives are *not* completely defaunated at this time as is the rule with all forms at each preceding molt. This has been determined by the examination of pre-adult reproductives from a few days before, and until, ecdysis. There is much less wood in the hind-gut of these forms and the total number of protozoa is diminished. The general behavior of these protozoa as observed in isolated reproductives at ecdysis and shortly thereafter, has shown that their survival is assured, however.

Pflugfelder (1947) has reported that the corpora allata increase in size in reproductive nymphs of *Microcerotermes amboinensis* and continue to enlarge for a time after the imaginal molt. This is not the case with soldiers and the so-called workers. Furthermore, in the sexual forms the growth of the ventral glands parallels that of the corpora allata until the final molt, at which time these glands degenerate. The corpora allata and ventral glands do not enlarge nor do the ventral glands degenerate, in the soldiers and workers. He considered the hypertrophy of the allata, at least, to be concerned with sexual metabolism.

The successful establishment of new colonies demands that each founding pair be provided with protozoa. It is quite possible that there may be neither time nor opportunity for these forms to obtain their infections in the usual manner after the final molt. Enormous numbers of these winged forms mature almost simultaneously, and the nuptial flight generally occurs within a short time thereafter. The observations presented in this paper, coupled with the findings of Pflugfelder, suggest that these obvious glandular changes, and the resulting shifts in hormone balance may constitute a mechanism for insuring the adult termites a supply of protozoa for their nuptial flight.

Summary and Conclusions

In the wood-feeding roach, *Cryptocercus,* most members of the 13 genera of intestinal flagellates change from asexual to sexual methods of reproduction each time their host molts. Members of one genus encyst some time before, and pass out with fecal pellets shortly after ecdysis. Since hatching is synchronized with molting, the young nymphs are thus enabled to acquire at least a part of their initial infections, which are then retained for life. The five flagellate genera found in the termite *Zootermopsis* undergo no sexual cycles and, with few exceptions, they all degenerate shortly before the molt. The termites therefore must refaunate themselves after each molt, and this they do by the solicitation of proctodaeal droplets containing protozoa from their non-molting associates. All protozoa may be removed from the roach and the termite with oxygen and without injury to either host. Reciprocal transfers of normal protozoan faunas have been made *per rectum* between these two insects.

The termite protozoa can live in and adequately maintain nymphal roaches for as long as 221 days, or probably as long as they do not molt. The protozoa can maintain adult roaches for at least as long as 245 days, or probably for the duration of adult life. However, examination of nymphal roaches one day or less before the molt showed that the vital majority of the protozoa became feeble, non-motile, and finally degenerated before this event took place. No evidence of sexual cycles was observed. These defaunated nymphs are behaviorally incapable of regaining an infection of termite protozoa under natural conditions and must shortly die of starvation. Thus, except under experimental conditions, termite protozoa could not indefinitely maintain a colony of *Cryptocercus.*

The roach protozoa, on the other hand, actually have supported individual termites for as long as one year, and through several instars. Some genera of the protozoa started sexual cycles in molting period termites, but these were all aborted and the termites were practically defaunated at ecdysis. But since they are naturally defaunated at this time, they were also able to regain infections of roach protozoa from other individuals so infected. In addition, second and third form reproductives containing roach protozoa have established small colonies in the laboratory, and have successfully reared nymphs through at least four instars. *Cryptocercus* protozoa are therefore very likely capable of supporting a colony of this termite indefinitely.

Various implications of these results are discussed, especially with regard to possibilities of determining the role of the host in initiating and controlling the sexual cycles of *Cryptocercus* protozoa.

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ON THE RELATION BETWEEN GROWTH AND MORPHOGENESIS IN THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM[1]

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In the normal course of cellular slime mold development, the myxamoebae pass through the exponential growth phase and, upon its cessation, aggregate in multicellular masses. The number of aggregative centers formed has been shown to be a function of the number of cells and of the population density (Sussman and Noel, 1952). From these and other relationships (Sussman, 1952) it can be inferred that each aggregate requires for its formation the presence of a special cell called an initiator, to which neighboring cells respond, and only a small proportion of the population can so serve under the conditions employed. In *Dictyostelium discoideum* wild type, the ratio of the initiator to responder cells has been found to be 1:2200. That the system is under genetic control is shown by the fact that mutants of *D. discoideum,* as well as wild type representatives of other species, display significantly different ratios (Sussman and Noel, 1952; Sussman, 1955).

In the past, Raper (1940) has presented evidence to indicate that aggregation can begin only when growth ceases and that the aggregation of a stationary phase population can be prevented and indeed reversed by further additions of nutrient material. The present study demonstrates an extension of this conclusion: that is, that cells taken from any phase of the growth cycle, including newly germinated spores, can aggregate when further growth is prevented and, in fact, give quantitatively the same aggregative performance as do stationary phase myxamoebae.

METHODS

A. Organisms and media. The organism used in this study is representative of *D. discoideum* Raper, strain NC-4. Stock cultures were grown on glucose-peptone agar in association with *A. aerogenes* by methods previously described (Sussman, 1951). Under these conditions the myxamoebae exhaust the bacterial supply after about 44 hours incubation at 22° C. and aggregate and construct fruits in the ensuing 24 hours. Spore suspensions from these fruits were used as innocula for special growth experiments to be described in the next section.

B. Aggregation experiments. All the methods and procedures employed have been described in detail elsewhere (Sussman and Noel, 1952). Washed myxamoebae were suspended in salt solution (Bonner, 1947), counted with a hemocytometer, and dispensed in drops on a washed agar-distilled water substratum. The excess fluid was absorbed by the agar leaving the cells randomly distributed within the confines of the drop. Under these conditions the amoebae remained constant in number, aggregated in normal fashion after 12 hours and produced

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[1] This study was aided by grants from the Office of Naval Research and The National Institutes of Health, U.S.PH.S.

typical fruits. Control of the number of myxamoebae delivered was achieved by appropriate dilution of the original cell suspensions. The population density of the cells on the agar was determined by the volume of the drop delivered.

RESULTS

A. The growth curve of D. discoideum on complete and restricted media

Spores, taken from a 4–6 day stock plate, were suspended in salt solution and spread on glucose peptone agar with 0.1 cc. of a 48-hour broth culture of *A. aerogenes*. The initial spore count per plate was 1 × 10^6. At intervals, duplicate plates were harvested using a finger and 10 cc. distilled water delivered in two batches. Control experiments using known numbers of cells indicated a recovery in excess of 95% of the cells on the plates. Replicate counts with the hemocytometer were made of each harvest. Figure 1 shows the results of two experiments (upper curve).

A technical difficulty precluded the use of this system for studying the aggrega-

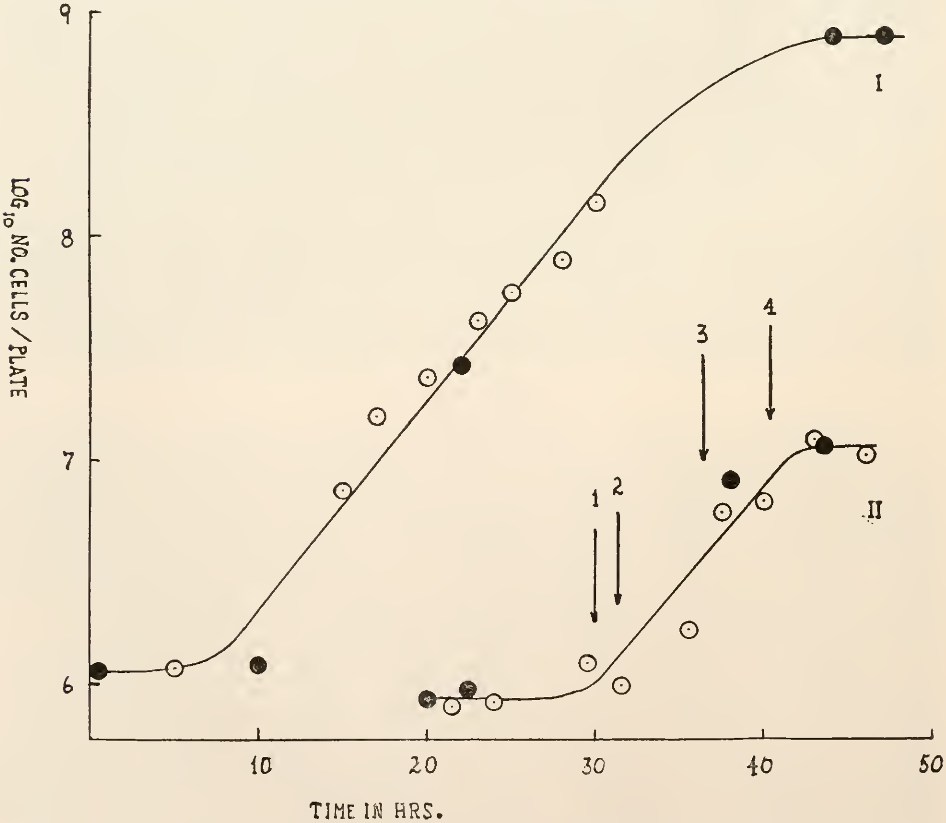

FIGURE 1. Growth curves of *D. discoideum* on complete (I) and restricted (II) media. The arrows indicate the times at which cell samples were taken for tests of aggregative performance.

tive performances of cells in different growth phases. During the log phase the number of bacteria was so great that clean preparations could not be obtained even by exhaustive washing in the centrifuge. The delivery of such contaminated suspensions to washed agar plates for the aggregation experiments led to considerable growth of the myxamoebae and rendered the results meaningless.

To avoid this hindrance, a restricted medium was used on which the bacteria remained in more convenient balance with the myxamoebae. A 48-hour broth culture of *A. aerogenes* was washed once in the centrifuge and suspended in water to ½ the original volume. One-half cc. aliquots were spread on washed agar plates with the spore inoculum and incubated at 22° C. Figure 1 (lower curve) shows the growth curve obtained from two experiments. The arrows show where samples of cells were taken for aggregation experiments to be described. The data reveal only minor differences in the growth curves on the two types of media. The lag phase, including the germination period, was about 7 hours on complete medium and about 11 hours on restricted. True exponential increases occurred on both media, the generation times being approximately the same (ca. three hours).

The stationary phase was attained at about 40 hours on complete medium and at 24 hours on restricted medium. A population of nearly 10^9 myxamoebae per plate on the former indicated a total of 9.5 generations of growth. On the latter medium the stationary population was slightly more than 10^7 per plate, the result of 3.8 generations of growth.

B. Aggregative performance of cells in different growth phases

As mentioned previously the number of centers of aggregation formed by a population of myxamoebae is a function of the number of cells and of the population density. The curve in Figure 2 demonstrates these relationships for samples of 10,000 washed myxamoebae taken from the stationary growth phase on glucose-peptone agar plates and dispensed on washed agar over a range of population densities. It is seen that at densities below 80 cells per mm.^{2} no centers appeared after 24 hours' incubation period. At high densities center formation was suppressed to some extent and at the optimal density, around 200 cells per mm.^{2}, a maximal number of centers was formed. The distribution of initiator cells to responder cells thus shown is 1:2100, in very close agreement with previous evaluations (Sussman and Noel, 1952). To determine the aggregative performances of cells in different growth phases, cultures grown on restricted medium were harvested at stages shown by the arrows in Figure 1. These stages were: 1 and 2, newly germinated myxamoebae at the end of the lag phase and/or at the beginning of the log phase; 3, at mid-log phase; 4, at the end of the log phase. The cells were washed by repeated centrifugation, suspended in salt solution, and replicate samples of 10,000 cells were delivered to washed agar over a range of population densities. It should be noted that even after thorough washing, the cells were contaminated to a slight extent with occluded bacteria. To avoid growth of the bacteria and therefore of the amoeba on the aggregation plates, streptomycin sulphate (Nutritional Biochemical) was added to the agar in final concentration of 0.5 gm. per liter. Unpublished experiments have shown that streptomycin prevents growth of the myxamoebae under these conditions but does not interfere in any observable manner with aggregation or fruit construction.

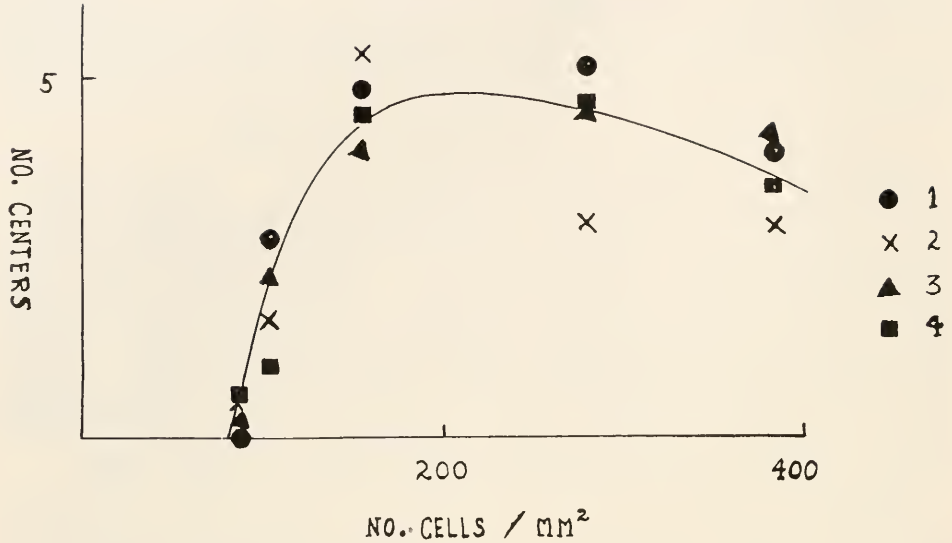

FIGURE 2. Relation between center formation and population density. Cell samples 1–4 were taken at times indicated by the arrows in Figure 1 and were washed by centrifugation and suspended in salt solution. Aliquots containing 10,000 cells were dispensed on washed agar over a range of densities. The densities were checked by direct observation with an ocular grid. The number of aggregative centers were counted after 24 hours. The ordinate shows the number of centers per aliquot.

A single experiment was performed at each of the stages mentioned. Figure 2 summarizes the data, each point representing the mean of at least six replicate samples. Aliquots containing relatively small numbers of cells (10,000) had to be used because of the practical limits imposed by the growth stages at which the cells were harvested. This magnified the random sampling error. Nevertheless, the points bracket the normal curve quite closely, and it is clear that no significant difference is apparent between the aggregative performance of cells in lag and log phases and that of cells from the stationary phase.

DISCUSSION

Evidence gained from a number of experimental directions has made it necessary to categorize aggregating myxamoebae as being either initiator cells that provide the aggregative stimulus or responder cells that answer it. One can account for this phenotypic divergence in at least two ways: a) The initiator cells arise spontaneously in the population via a stable genetic alteration. The ratio of initiator to responder cells is governed by selective pressures of the environment during the growth of the population. b) The matrix of conditions attending the cessation of growth induces a small proportion of the population to attain initiative capacity above the threshold necessary to attract responder cells under the conditions employed. This involves no prior genetic alteration but merely results from the exploitation of random physiological differences among the cells at that time. Thus, prior to the stoppage of growth, there are no initiator and responder cells *per se.*

The experiments described here support the second of these alternatives. In the mature fruiting body the spores represent a non-random sample of the original myxamoeboid population. This follows from the fact that the fates of the myxamoebae (whether they are to become spores, stalk cells, or basal disk cells) are determined by the order in which they entered the aggregates. The prestalk cells enter first, prespore cells next, and prebasal disk cells last (Bonner, 1944). Were the difference between initiator and responder cell due to a persistent, spontaneous, genetic change, one would expect that a newly germinated spore population should display a skewed distribution of initiator cells and that, during the subsequent growth of these cells, selective conditions would systematically alter the distribution until the customary ratio of 1:2200 was regained. In contrast, the second alternative does not carry with it the necessity of presuming these fluctuations in the initiator: responder ratio during the growth cycle. If the passage from a stage of active growth and synthesis to the stationary state itself evoked the random physiological variation that in turn determined whether a cell was to be an initiator or responder, then the distribution of initiators might be the same no matter what the past history of the cells.

The view is in accord with previously published results (Sussman, 1952, 1955) that indicate a spectrum of initiative capacity in the population. Thus in any system, the number of centers actually formed would depend both upon this spectrum and upon the level of sensitivity of the responder cells.

SUMMARY

1. The growth kinetics of *Dictyostelium discoideum* have been determined on complete and restricted media.

2. Myxamoebae were taken from different parts of the lag and log phases and allowed to aggregate under conditions permitting quantitative examination. No difference could be discerned between the aggregative performance of these cells and that of stationary phase myxamoebae with respect to the number of centers formed or the population density response.

3. The bearing of these results upon the mechanisms of cellular differentiation during slime mold development is discussed.

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STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] I. INHIBITION OF CYTOCHROME OXIDASE BY ULTRAVIOLET

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The effect of radiations of various types on enzyme systems has been the subject of numerous investigations. The exemplary work of Dale (1940, 1942), and of Barron and his associates (1949) has indicated that many enzymes are readily inactivated by ionizing radiation when sufficiently pure and when in dilute solution. Furthermore, their work clearly demonstrated that molecules, other than enzyme, when present in the irradiated solution, would protect the enzyme from inactivation. The current work on radiation "protection" has its origin in these findings. Such investigations have led to a clear picture of the action of ionizing radiations in the inhibition of various enzyme systems and have directed many investigators to seek the cause of gross radiation damage to organisms at the enzyme level. Similarly, much has been done to elucidate the nature of ultraviolet radiation damage to enzymes (McLaren, 1949; Lea, 1947). As early as 1897, Green had reported the destruction of diastase by ultraviolet. Nevertheless, less attention has been paid to the role of enzyme inactivation in lethal changes induced in cells by ultraviolet radiation. This is largely the result of the close correspondence of the nucleic acid absorption spectrum and most action spectra in the ultraviolet.

Although the disparity between the studies of "genetic" damage and enzyme damage is explicable in terms of the action spectra, it is surprising that in spite of the vast amount of work on the effects of radiations, little has been reported concerning their effects upon the particulate systems of cells. There are only two reports (both negative) of the effects of x-radiation on the cytochrome oxidase activity of particulates (Barron *et al.*, 1949; LeMay, 1951), and one report of the loss of the cytochromes from yeast after ultraviolet radiation (Raut, 1954). When one considers the vital role which has been assigned to particulates in the cellular metabolism, it would seem that any damage to this system would result in rather drastic changes in the cell. The remarkable similarity of the enzyme content of the particulates and the enzymatic mechanisms involving aerobic metabolism in widely differing organisms suggests a role in the continuity of the cell similar to that played by the chromosomes. For these reasons, it seemed advisable to make a study of the effects of various types of radiations on the mitochondrial fraction of the cellular particulates. The effects of "germicidal" ultraviolet radiation are reported in this paper.

For a test agent, cytochrome oxidase was chosen. This was done for three reasons: a depression in oxygen uptake has been observed by many workers following irradiation, cytochrome oxidase is closely bound to the particulate and there-

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fore clearly forms part of its structure, and the recent reports of the enhanced effect of x-radiation after the addition of CO and CN (King, Schneiderman and Sax, 1952) indicate a possible role of cytochrome oxidase in radiation damage. Furthermore, cytochrome oxidase has been shown to be the terminal oxidase of many different organisms, and it seems evident that damage to this enzyme would result in alterations in the cellular metabolism of a very basic nature.

MATERIALS AND METHODS

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where a good deal of the blood was washed free. The liver was weighed and pressed through a bronze sieve to remove connective tissue. The resulting mash was then suspended in cold 8.5% sucrose (50 ml.) and homogenized in a glass homogenizer with a "Teflon" pestle driven by a cone-drive stirring motor. The mitochondria were then separated from the rest of the homogenate by the method of differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept. This fraction was washed twice with cold 8.5% sucrose, and the final sediment was suspended in 2.5 ml. sucrose solution per gram of liver. All centrifugations were carried out in a refrigerated centrifuge at 4° C.

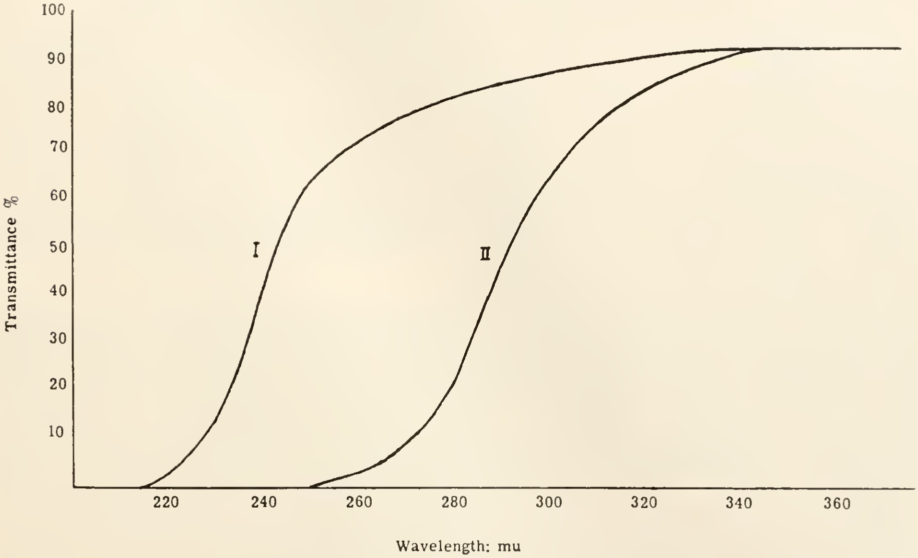

FIGURE 1. The transmission of the two filters used in this study. Filter I is Corning No. 7910 and Filter II is Corning No. 9700. Curves drawn from data supplied by the Corning Glass Co. and spot checked with a Beckman spectrophotometer.

For irradiation, a sample of the suspension was diluted 1 in 5, 1 in 10, or 1 in 15 with the same sucrose solution. (The dilutions used in each case are given with the data.) Two and five-tenths ml. were placed in a quartz flask rotated in the ultraviolet beam by a slow speed stirring motor; 2.5 ml. were kept in a Pyrex test tube, in the cold, as a control. During the period of irradiation, the quartz flask was partially submerged in an ice-water bath maintaining a temperature under 5° C. The flask was kept 11.5 or 18.5 cm. from a G.E. germicidal lamp for all radiations. The 18.5-cm. position is referred to as the low intensity position in the rest of the paper. The intensity of the radiation was 5500 ergs/cm.^{2}/sec. at the 11.5-cm. position as measured by a G.E. light meter with ultraviolet cell. In the filter studies, different filters were placed between the source and the flask. The transmission of the two filters used is given in Figure 1. Filter I is a Corning ultraviolet transmitting filter (No. 7910) which transmits principally those wave-lengths above 2400 Å; and Filter II (Corning No. 9700) transmits principally those wave-lengths above 2800 Å. In a few experiments, the mitochondrial suspension was irradiated in an open petri plate to test the effect of the rotation in the quartz flask. When this was done, controls were also kept in the cold in an open petri plate.

In the studies done with whole cells, cells of *Parthenocissus* crown gall grown in tissue culture were used. These cells were spread out on moist filter paper in a petri dish and exposed to the ultraviolet radiation as above. The cells were then collected and the mitochondrial fraction obtained in the same manner as that used for rat liver, with the exceptions that the tissue was ground with sand in a mortar and the final suspension was made up in a total of 2 ml. sucrose. The small fraction obtained in this way showed cytochrome oxidase activity. In cases where the oxygen uptake of whole cells is reported, the results represent the endogenous respiration. All radiations of whole cells were carried out in the same way and at the same distance (11.5 cm.) from the source.

The cytochrome oxidase activity was estimated manometrically by the method of Hogeboom, Claude and Hotchkiss (1946). The main compartment of each vessel contained: 0.35 ml. of the mitochondrial suspension, 0.1 ml. Sorenson phosphate buffer (pH 7.4), 1 ml. 1.3×10^{-4} M cytochrome-*c* (Sigma, horse-heart) in 0.85 % NaCl, and 0.15 ml. 0.005 M $AlCl_3$. The center well contained 0.2 ml. 5 N KOH; and the side arm held the reducing agent, 0.15 ml. 0.228 M sodium ascorbate. In studies concerning the effects of added nucleotides, each flask contained 5 μM AMP (adenosine-5-phosphoric acid, free acid—Sigma), ADP (adenosine diphosphate, sodium salt—Sigma), or ATP (adenosine triphosphate, neutral sodium salt —Schwartz). In these cases, as in the case of the determination of the effect of pH on the enzyme, the buffer in the flask was changed. In the experiments with the nucleotides the buffer was increased in an attempt to keep the final pH of the reaction at 7.4. In the pH series, various phosphate buffers were used to give the desired range. In the determination of the oxygen uptake of the whole cells the vessels contained only cells and one ml. of water. The center well contained alkali as above. All determinations were made at 38° C. with the exception of the whole cells and the ones made with ATP. These two latter were done at 30° C.

Attempts to photoreactivate the system were made by placing the irradiated suspension under two 250-watt fluorescent lights for various lengths of time, as indicated in the tables. In some cases the exposure to light was made in an ice bath and in others the experiments were carried out at room temperature. The suspension was exposed to the light in a petri plate with the cover left on, and with two thicknesses of window glass between the petri plate and the source. This was done to remove any ultraviolet radiation from the fluorescent tubes.

All assays were made in triplicate. In addition, most experiments were repeated three or more times; some of the less important points on the dosage curve were repeated only twice.

RESULTS

The data presented in Table I show the effectiveness of ultraviolet radiation in the inactivation of cytochrome oxidase. In this case, representative experiments are shown (chosen to correspond to the average value for several runs). It can be seen that 20 minutes of radiation are sufficient to reduce the oxidase activity to about 40% of the controls, and that at one hour only 20% of the activity is left. These values were obtained using Filter I; without the filter the source is only slightly more effective, the difference being due to the absorption of the filter. Table I also presents data on the effect of rotating the suspension in the quartz flask without radiation. This was done to see if there was an inactivation of the oxidase by this procedure. This particular experiment was repeated ten times, and there is an average inactivation of 8.2% with one hour rotation. This is very slight when compared to the effect of the radiation and can be ignored for most of the studies (wherever Filter I is used).

TABLE I

The effect of ultraviolet radiation and of rotation upon cytochrome oxidase activity

(Preparation diluted 1 : 5)

Treatment	μL O_2	Per cent inactivation
Control	365.4	
20 min. UV	163.6	55.2%
Control	159.4	
60 min. UV	29.9	81.2%
Control	134.7	
60 min. rotation	124.0	7.9%

Table II presents the data obtained when different filters were used. The activity is expressed as per cent of the controls and represents an average value of all experiments. It is evident that Filter I (wave-lengths above 2400 Å) is much more effective than Filter II (wave-lengths 2800 Å). One hour of radiation with Filter

TABLE II

Relative effectiveness of filtered ultraviolet radiation

(Preparation diluted 1 : 5)

Filter*	Treatment	Per cent control activity
I	4 min.	80.5%
I	10 min.	63.8%
I	60 min.	19.0%
II	60 min.	81.8%
II	180 min.	62.5%

* Filter I transmits mostly wave-lengths above 2400 Å; Filter II transmits mostly above 2800 Å (see Fig. 1).

II produced about as much inactivation as 4 minutes with Filter I; three hours with Filter II is equivalent to 10 minutes with Filter I. It seems that Filter II is only about $\frac{1}{15}$ as effective as Filter I (about 6%).

Figure 2 presents the relation between the inactivation of cytochrome oxidase and the dosage of ultraviolet. The curve was obtained using Filter I. This curve is a composite and should be considered only as a rough indication of the course of inactivation. All the points represent average values from several experiments. There is some variability expected in this case, since if one uses the same rat liver for all points on the curve, the controls are slowly losing activity with age, and if one uses different preparations for the different points, there is variation in the cytochrome oxidase concentration of the initial suspensions. However, it is clear that the initial inactivation of the enzyme is rapid, but the slope of the curve becomes more gradual after 20 minutes, reaching a maximum inactivation at about one hour. This curve is similar to the inactivation curves obtained with microorganisms, and the shape is probably due to the gradual increase in protection against radiation by the inactivated enzyme. At the high intensity of radiation used in these experiments, it was not possible to determine the threshold value for the inactivation. Therefore the intensity was reduced by moving the flask away from the source. The data obtained with this lower intensity radiation are presented in Figure 3. It is evident that there is a definite threshold value for the inactivation, and that this value varies with the age of the preparation. There is distinct evidence for a slight increase in the activity of older preparations when given very low doses of ultraviolet. With higher doses (as in Figure 2) there is never any indication of an increase in activity.

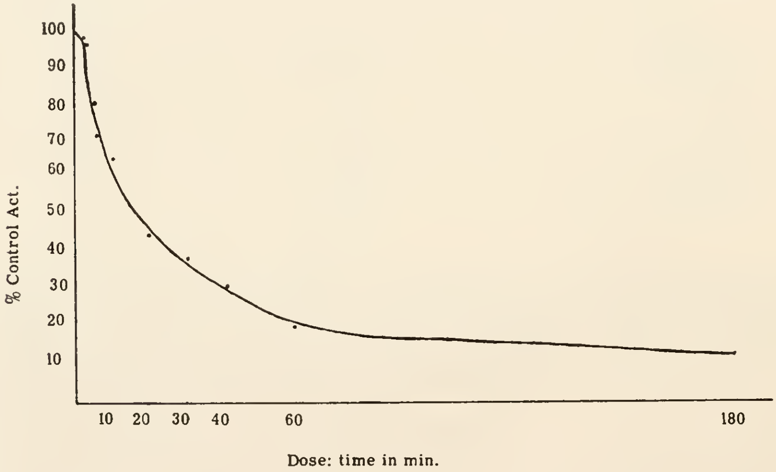

FIGURE 2. Inactivation of cytochrome oxidase by ultraviolet radiation. Radiation at high intensity; filter I; preparation diluted 1 in 5. Activity is expressed as per cent of the control activity.

Table III presents data obtained in attempts to reactivate the irradiated cytochrome oxidase. It is evident that there is neither photoreactivation nor reactivation with ADP or ATP. During the attempts to reactivate the preparation with ADP and ATP, a slight stimulation of both the controls and the irradiated preparation by the ADP and a slight inhibition by ATP was noted. It was thought that this might be an indication of the participation of these compounds in the oxidation of cytochrome-*c* as suggested recently by the work of Lehninger, Hassan and Sudduth (1954). However, it is extremely difficult to maintain a constant pH in the vessels during a test made with these compounds, and subsequent investigation indicated that the enzyme is extremely sensitive to slight variations in pH. The relationship between cytochrome oxidase activity and pH is given in Table IV. There seems to be no effect of the added ADP or ATP which is not the result of a shift in the pH. These data seem to indicate that it is not the adenylic acid system which is being inactivated by the ultraviolet. The absence of photoreactivation in such a simple system is not surprising.

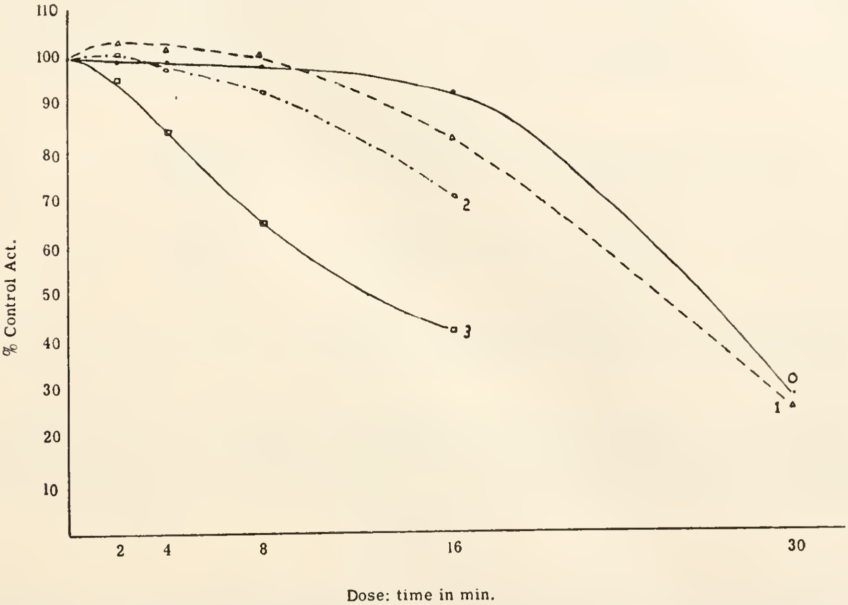

FIGURE 3. Inactivation of cytochrome oxidase preparations of varying ages by ultraviolet radiation. Source at lower intensity position; filter I; preparation diluted 1 in 15. ·———· preparation radiated same day extracted; △---△ preparation radiated one day later; ○—·—○ preparation radiated two days later; □——□ preparation radiated three days later.

To determine the effect of ultraviolet radiation on the cytochrome oxidase activity of irradiated whole cells, cells of *Parthenocissus* crown gall were irradiated and the particulate fraction isolated after radiation. The data from these experiments are summarized in Table V. It can be seen that although 5 minutes of radiation reduces the endogenous oxygen uptake of the intact cells 17%, it takes one to two hours to achieve equivalent inactivation of the particulates removed from the irradiated cells. It would seem that the cytochrome oxidase of these cells is not greatly affected by ultraviolet radiation so long as the radiation is carried out on

TABLE III

Failure to reactivate irradiated cytochrome oxidase

(Preparation diluted 1 : 5)

Treatment	% control activity
10 min. UV	65.6%
10 min. UV + ADP	67.2%
10 min. UV	66.3%
10 min. UV + ATP	61.4%
20 min. UV	44.8%
20 min. UV + ADP	46.3%
20 min. UV	44.6%
20 min. UV + ATP	39.8%
(Preparation diluted 1 : 10)	
40 min. UV	36.7%
40 min. UV + 20 min. light*	33.2%
2 min. UV	74.4%
2 min. UV + 20 min. light†	70.0%
20 min. light†	94.2%

* Continuous light; treatment at 5° C.

† Two 10-minute light periods separated by 10 minutes of dark; treatment at room temperature.

the intact cells. Control experiments indicate no difference in the sensitivity of particulates radiated after being extracted from the cells. It is apparent that the cytochrome oxidase is protected by the rest of the cell from the radiation damage.

DISCUSSION

The data presented above indicate that cytochrome oxidase in the isolated cellular particulate is very sensitive to ultraviolet radiation. Furthermore, these data indicate that ultraviolet wave-lengths shorter than 2800 Å produce this effect. Reference to Table II indicates that Filter II is only about 6% as effective as Filter I. At these low values of inactivation, the effect of rotation of the quartz flask becomes appreciable. Table I shows that an hour of rotation decreases that activity of the preparation about 8%. Therefore, one may assume that the value of rela-

TABLE IV

The effect of pH on the activity of cytochrome oxidase

(Preparation diluted 1 : 10)

pH	Oxygen uptake (μL O_2)	% activity of 7.4
7.4	195.6	100.0
7.3	177.0	90.5
7.2	141.8	72.5
7.1	127.4	65.1
7.0	115.0	58.8

tive effectiveness given for Filter II is on the high side. From a comparison of the transmission of the two filters (Figure 1), and from a comparison of their relative effectiveness, it would seem that the effect is induced by wave-lengths in the region 2600–2700 Å. However, the source used in these studies was a germicidal lamp, and therefore most of the emitted energy was in the region of 2537 Å. One would expect a protein to be denatured by all wave-lengths below 3100 Å, with maximum effects at the absorption maxima of the protein. To test the efficiency of the source and filters on the inactivation of a protein, crystalline urease prepared by the method of Sumner (1926) and Dounce (1941) was irradiated in the same manner as the cytochrome oxidase. It was found that with Filter I, 10 minutes of radiation were necessary to produce 50% inhibition of the enzyme; to achieve similar inactivation required 60 minutes of radiation with Filter II. Therefore, it is evident that for the inactivation of a more simple protein molecule, urease, the filters have an efficiency ratio of 6. Since urease has been reported to have an absorption maximum typical of simple proteins in the region of 2800 Å, and since the action spectrum for destruction of the enzyme and the absorption spectrum coincide (Kubowitz and Haas, 1933), this efficiency ratio provides us with a measure of the radiation from this source in the region 2800 Å. Since the destruction ratio for cytochrome oxidase is 15:1 with these same filters, it must be concluded that the wave-lengths below 2800 Å are about two times as efficient as those above. Therefore it seems possible to conclude that the cytochrome oxidase activity of isolated particulates is readily inactivated by wave-lengths in the region of 2600–2700 Å. Since some effect is obtained with Filter II, the wave-length cannot be lower than 2600 Å.

TABLE V

The effect of irradiation of whole cells upon the oxygen uptake of the cells and of isolated particulates

Test	Treatment	Oxygen uptake (μL O_2)	% inactivation
Cells	Control	48.4	
	5 min. UV	40.4	17%
Cells	Control	48.3	
	120 min. UV	20.8	57%
Particulates	Control	48.1	
	60 min. UV	43.9	9%
Particulates	Control	56.6	
	60 min. UV	47.1	17%
Particulates	Control	49.2	
	120 min. UV	43.7	11%

The fact that wave-lengths in the vicinity of the so-called "nucleic acid peak" are capable of deactivating cytochrome oxidase suggested that some nucleotide associated with the activity of the oxidase was being destroyed. Recent work by Lehninger, Hassan and Sudduth (1954) has demonstrated the formation of ATP during the oxidation of cytochrome-*c*. It was thought that perhaps either ADP or ATP was destroyed by the radiation and that this destruction was acting to inhibit the over-all reaction. Attempts were made to reactivate the preparation by the addition of both of these compounds, without success. It is possible that there is a nucleotide bound to the enzyme, however, which is destroyed and cannot be replaced by external sources; unfortunately such an hypothesis is difficult to test. As pointed out above, in the course of these investigations it was discovered that the pH had a very great effect upon the enzyme activity, more than could be accounted for by changes in the cytochrome-*c*. It is possible that the ultraviolet acts upon the particulate in such manner that the internal pH is shifted. However, this seems unlikely when the effect remains after prolonged exposure to buffer at the optimal pH.

It might also be suggested that the ultraviolet is destroying the structure of the particulate and thus inactivating the enzyme. This seems unlikely in view of the fact that the enzyme can be solubilized and retain its activity (Eichel *et al.,* 1950). Experiments done with the solubilized preparation indicate that the oxidase is inactivated by ultraviolet radiation in the same manner as in the intact particle. This also seems to eliminate the possibility that the effect is upon some other enzyme in the oxidative pathway, whose loss inhibits cytochrome oxidase. The best assumption, at present, seems to be that the effect is upon the oxidase molecule directly. When the data presented in Figure 3 are considered, it becomes evident that at low doses there is a stimulation of oxygen uptake, under certain conditions. This effect is observed most readily in a preparation that has aged for one or two days in the cold. Most frequently this effect is associated with an increase in the activity of the controls, when compared to those of the preceding day. The simplest explanation of this "aging" effect is that the permeability of the particulate membrane is increasing with time. The effect of the ultraviolet radiation may be to hasten (or increase) this change. It should be noted that this effect is not limited to old preparations. Sometimes it is observed with fresh preparations, generally with very low doses. Apparently this effect depends upon the conditions which prevail in the cell before extraction. It will be noted that the increase with low doses of ultraviolet disappears as the preparation gets older. At this time the control activity is also dropping, indicating a loss of activity by the cytochrome oxidase, for which further shifts in permeability would not compensate. These observations may provide an explanation for the reports of stimulation of oxygen uptake by ultraviolet radiation in whole cells. In the whole cell, as can be seen in Table V, the oxidase molecule is protected by other cellular constituents. This represents an effective lowering of the dose of ultraviolet, and may, under some conditions, result in a stimulation of the activity of cytochrome oxidase. It is evident that the effect of the ultraviolet in increasing the activity of the cytochrome oxidase may not be an effect on the permeability of the particulate membrane; however, since the effect becomes more pronounced with age, it seems to be an effect on something other than the oxidase molecule itself, permeability being the most likely suspect.

It is evident that further analysis depends upon a determination of the absorption spectrum of the cytochrome oxidase molecule and an elucidation of the action spectrum for its inactivation. Neither of these problems is simple. Measurements of the absorption spectrum of a suspension of isolated particulates show no clear peaks in the ultraviolet below 2800 Å. There is a general peak between 2800–2900 Å, corresponding to the protein maxima, but below this the absorption tapers off gradually. The difference in the absorption between 2800 and 2600 Å is rather small, indicating general absorption in the ultraviolet. Attempts were made to concentrate the cytochrome oxidase in its solubilized form by the method of Wainio *et al.*, (1948). Isolation of the enzyme resulted in a clear (yellow) preparation having reasonable activity. However, the preparation is completely opaque to wavelengths below 2700 Å, largely the result of the extractant, desoxycholic acid. Removal of the extractant results in the precipitation of the enzyme in an insoluble form. Further work in this direction awaits a new method for the purification of cytochrome oxidase. The only indication that the molecule has an absorption peak at 2600–2700 Å is the CO spectrum obtained by Warburg and Negelein (1929). They obtained a strong peak in this region. Measurements of the action spectrum for the inactivation of the enzyme are complicated by the fact that the intensity of most monochrometers is too low to give meaningful results. The radiation time becomes excessive, and the preparation is destroyed by other factors than the radiation. A sufficiently intense monochrometer is not available in this laboratory at the present time.

The fact that ultraviolet radiation in the region 2600–2700 Å inhibits cytochrome oxidase activity suggests that one of the causes of cell death may be the destruction of this enzyme. It is well known that ultraviolet radiation in relatively low doses blocks the cell division mechanism, and at higher doses causes the death of cells. This effect has been attributed to a change in the nucleic acids of the cells. It seems quite possible that the effect is on the enzyme systems of the cells. Inactivation of one of the major terminal oxidases would undoubtedly disturb the metabolism of the cell and possibly result in death. Attempts were made to test this idea using cells of *Parthenocissus* crown gall grown in tissue culture. These cells were chosen because they can be grown in large quantities, and they lack the pigments which would interfere with the absorption of the ultraviolet. Attempts were made to study this problem with rat liver cells, but even with repeated washing of the cells they could not be totally freed of blood. The plant cells seemed to be ideal. The cell division mechanism is inhibited with relatively low doses of ultraviolet (two minutes), and the respiration is also inhibited. However, as can be seen in Table V, these effects cannot be attributed to an effect upon the cytochrome oxidase. It would appear that the effect of the ultraviolet is upon some step preceding the terminal oxidase or upon some other enzyme acting as a terminal oxidase in these cells. As cytochrome oxidase may not be the major terminal oxidase, the test is inconclusive. Tissue which is normally exposed to ultraviolet radiation and which is sufficiently clear to permit penetration (skin, cornea, etc.) is difficult to come by in sufficient quantities to extract the particulates. At the present time no final conclusion can be reached on this point. It does seem probable that exposed tissues lose the activity of cytochrome oxidase in cells in which this is a major oxidase. The consequence of this loss may be drastic or slight depending upon the degree of destruction of the enzyme and the other mechanisms present in the cell to carry on the oxidative metabolism.

Summary

Data are presented which indicate that ultraviolet radiation of 2600–2700 Å is effective in the inactivation of cytochrome oxidase in intact cellular particulates. The effect seems to be upon the oxidase molecule itself. It is suggested that this could be one cause of cell death from ultraviolet radiation.

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MOLTING AND SHELL GROWTH IN BALANUS AMPHITRITE NIVEUS[1]

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The frequency of molting over an extended period of time has not been determined for any barnacle except *Balanus improvisus* (Costlow and Bookhout, 1953). Previously a few estimates had been made on barnacles of unknown age for limited periods only. Darwin (1854) mentioned that Mr. W. Thompson of Belfast maintained 20 specimens of *Balanus balanoides,* for 12 days, presumably in in the laboratory, and on the 12th day all had molted once and one individual twice. Thomas (1944) reported that *Balanus perforatus* molted every fifth day but did not give the age or the length of time these were observed. Costlow and Bookhout (1953) have shown that *B. improvisus* maintained in the laboratory molt every 2–3 days. Several series were followed over various lengths of time, the longest being 120 days during which time the individuals underwent 42 molts. Shell growth was continuous but erratic, and there was no indication that it was related to the molting cycle.

Since *Balanus improvisus* molts more frequently than was suspected by Darwin (1854) and Thomas (1944), and is primarily a winter barnacle at Beaufort, N. C., it may or may not be typical of other species. Therefore, it was deemed advisable to make similar studies on *Balanus amphitrite niveus,* a barnacle which settles during the summer and is most commonly found in the intertidal region at Beaufort. The purpose of this investigation was to determine the molting frequency, period of intermolt, relationship of shell growth to the molting cycle, and the effect of different light conditions.

METHODS

One-day old barnacles were collected by suspending plastic squares 6″ × 6″ in the Beaufort Harbor for 24 hours. Small pieces of plastic containing one pinhead barnacle each were cut from the large squares and placed in plastic compartmented boxes. The diet consisted of *Chlamydomonas sp.,* a type of food which supported the best and most consistent growth in *B. improvisus.* All series were maintained in a culture cabinet which was lighted with daylight fluorescent lamps at a tempera-

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Table I

Culture conditions for eight series of Balanus amphitrite niveus

Series	Original number	Final number	Days maintained	Light period	Purpose
I	107	18	47	24 hrs. light	molting
II	27	13	47	24 hrs. light	molting and shell growth
III	20	12	18	24 hrs. light	molting and shell growth
IV	35	19	25	24 hrs. darkness	molting
V	32	19	24	24 hrs. darkness	molting and shell growth
VI	40	10	22	12 hrs. light 12 hrs. darkness	molting
VII	20	11	18	12 hrs. light 12 hrs. darkness	molting and shell growth
VIII	56	37	14	natural environment	shell growth

ture of approximately 20° C. The barnacles and surrounding medium were examined daily to determine if a molt were present. The old culture medium was replaced by fresh at this time. Each barnacle studied for growth was measured daily by the use of an ocular micrometer and the areal increment of the basis computed from these measurements for the 24-hour period.

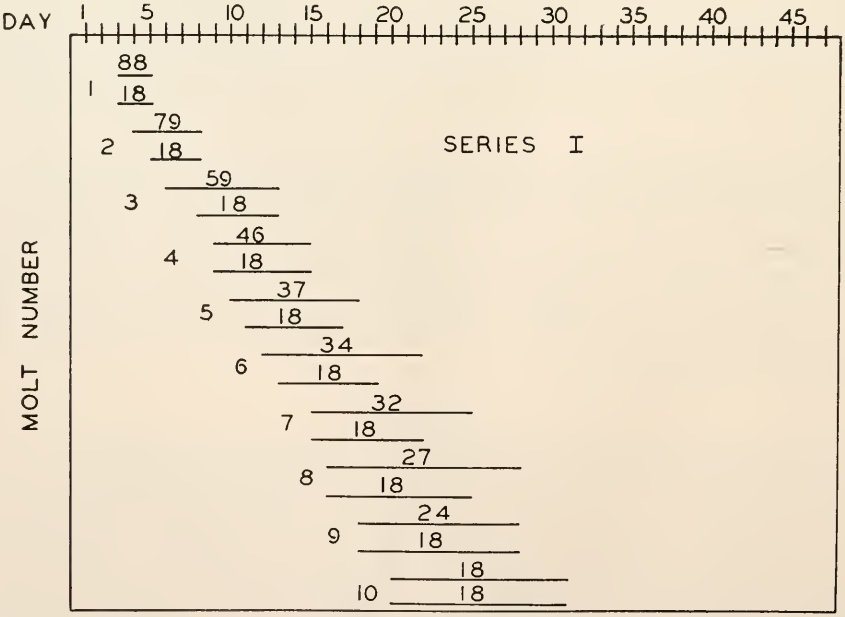

Figure 1. Molting periods of *Balanus amphitrite niveus* when reared in continuous light. Series I. The first line indicates the range of time for one molt and the number of molts for entire series. The bottom line indicates the range of time for one molt and number of molts for those individuals which lived throughout the entire period.

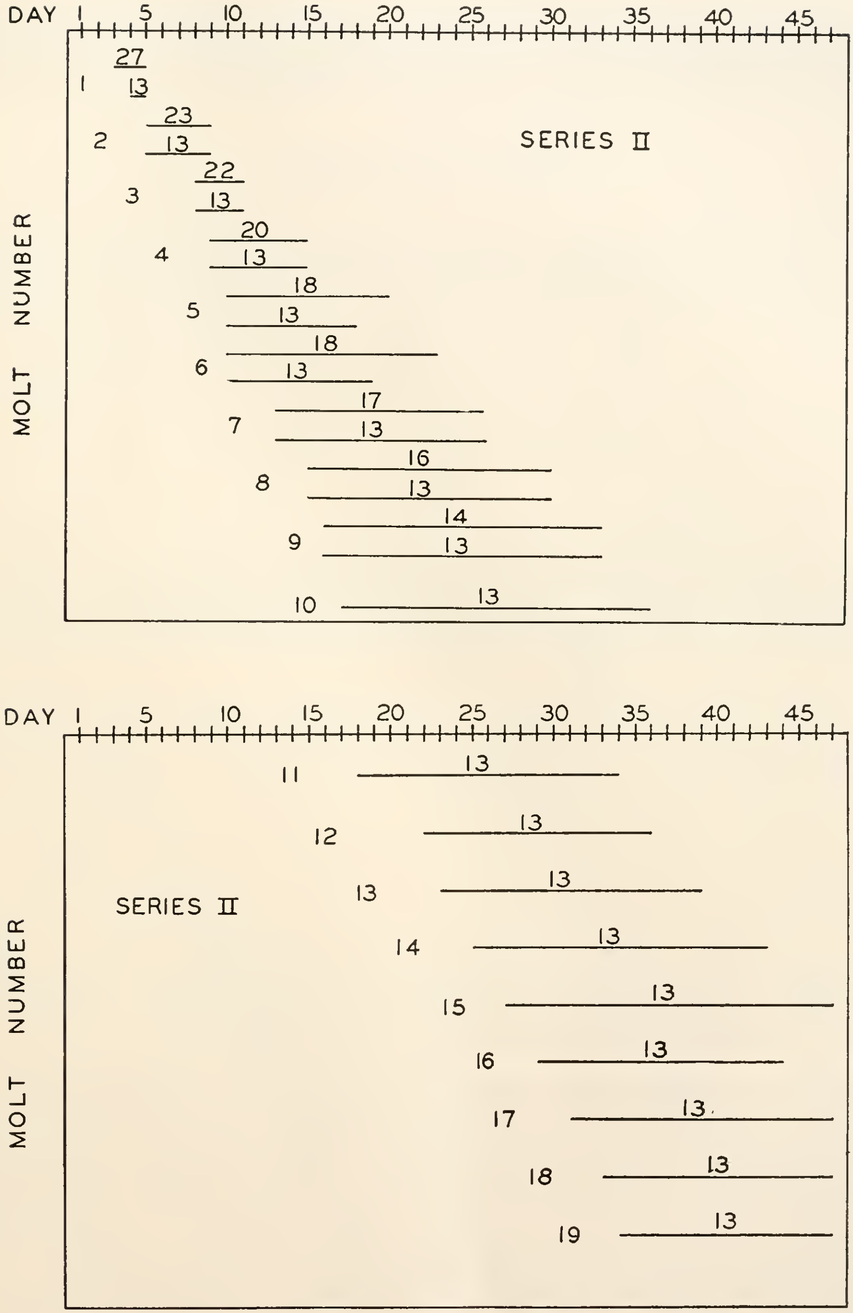

FIGURE 2. Molting periods of *Balanus amphitrite niveus* when reared in continuous light. Series II. The first line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

Table I gives pertinent data concerning the number of series and the conditions under which they were raised. In addition to the numbered series, two series were cultured in autoclaved, filtered sea water with no food source, to accurately determine the time of the first molt.

Results and Discussion

Molting

The results of the comparative study of molting are given in Figures 1, 2, and 3. It may be noted that the time of molting for each of the series is relatively uniform for the first five molts, but from the sixth molt through succeeding ones there is considerable variation which resembles the condition in *B. improvisus* after the ninth molt. From a study of the individual records of the actual time of molting it is found that the average intermolt period varies from 2–3 days, an interval which agrees with that determined for *B. improvisus*. Extreme variations from this mean were noted within each series, even though they were maintained under identical conditions. For example, some barnacles molted eight times on eight successive days, whereas, within the same series others showed an intermolt period of 5 days over a period of two to three molts.

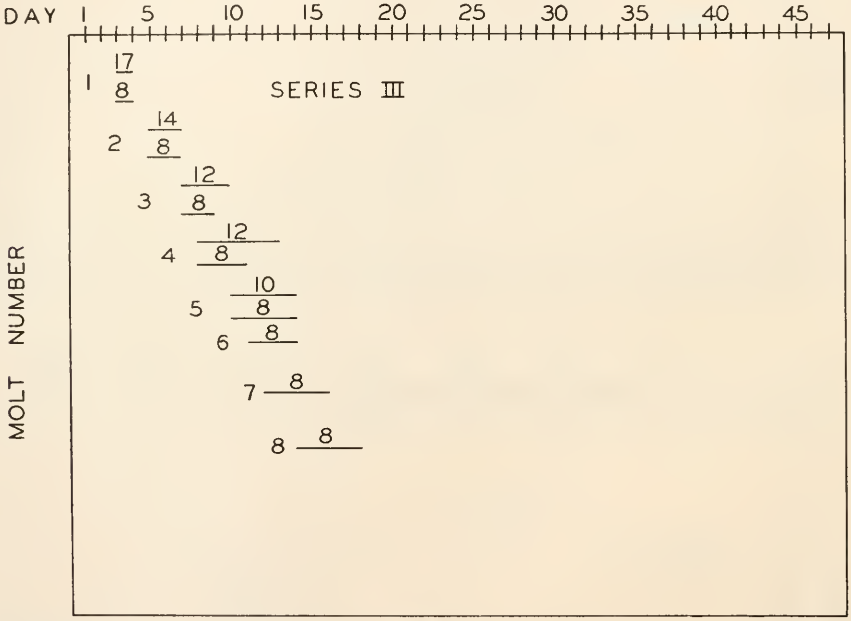

Figure 3. Molting periods of *Balanus amphitrite niveus* when reared in continuous light. Series III. The top line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

Figures 4, 5, and 6 show the molting periods for the series reared in total darkness and the series raised in twelve hours of light and twelve hours of darkness. Figure 7 gives a statistical analysis of the molting frequency of barnacles in all

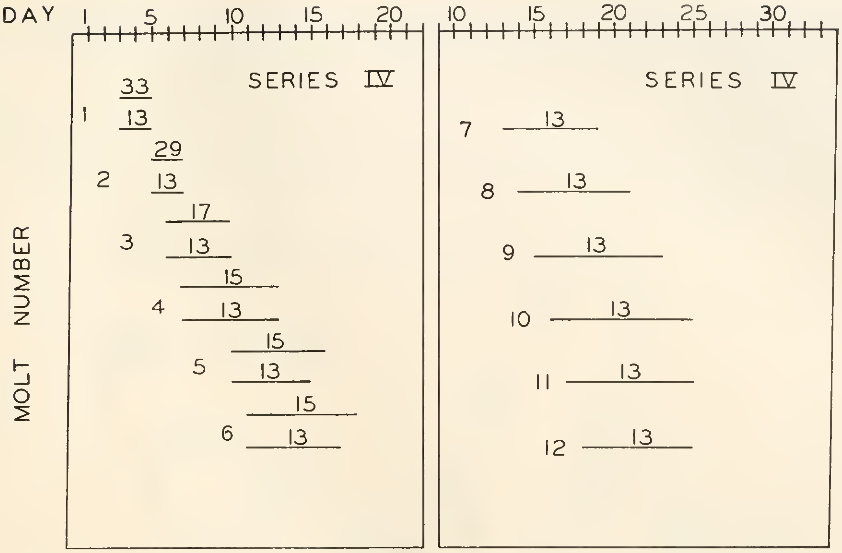

FIGURE 4. Molting periods of *Balanus amphitrite niveus* when reared in continuous darkness. Series IV. The top line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

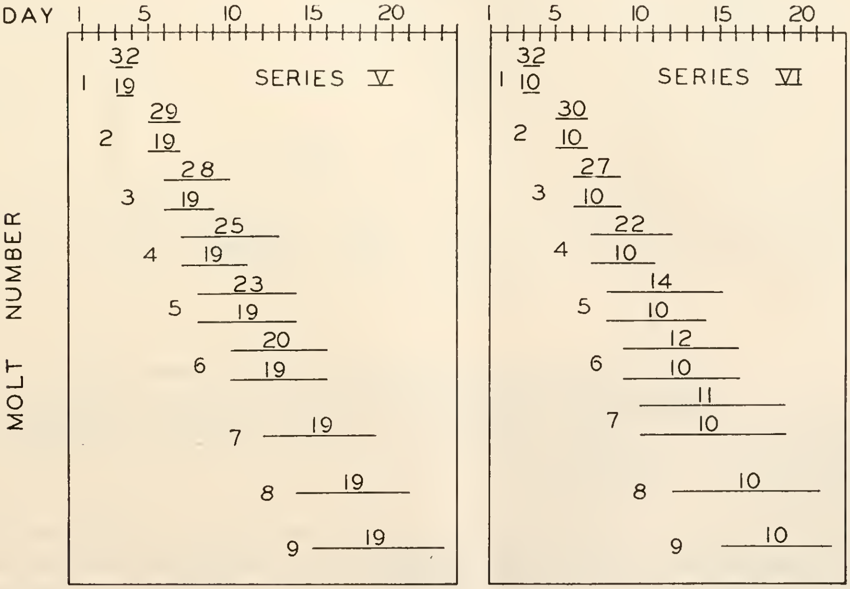

FIGURE 5. Molting periods of barnacles of Series V and VI. Series V was cultured in continuous darkness and Series VI in twelve hours of light and twelve hours of darkness. The top line indicates the number of molts for entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

three conditions of light. From an examination of Figure 7 it would appear that barnacles grown in total light, total darkness, or twelve hours of light and twelve hours of darkness exhibit essentially the same molting frequency.

In *B. improvisus* (Costlow and Bookhout, 1953) the first molt was overlooked because of the minute size of the exuviae and the masking effect of the food present. The 2–3 day frequency of molting indicated that an earlier molt might have been missed. In order to determine if this were the case a series of 10 barnacles was reared without food and was examined daily under the higher powers of the microscope. On the second day nine had molted and the tenth died in the process of molting. This procedure was repeated several times for *B. amphitrite niveus*, but all barnacles died within the first two days without molting. Thus it would appear that *B. amphitrite niveus* will not live nor pass through the first molt without

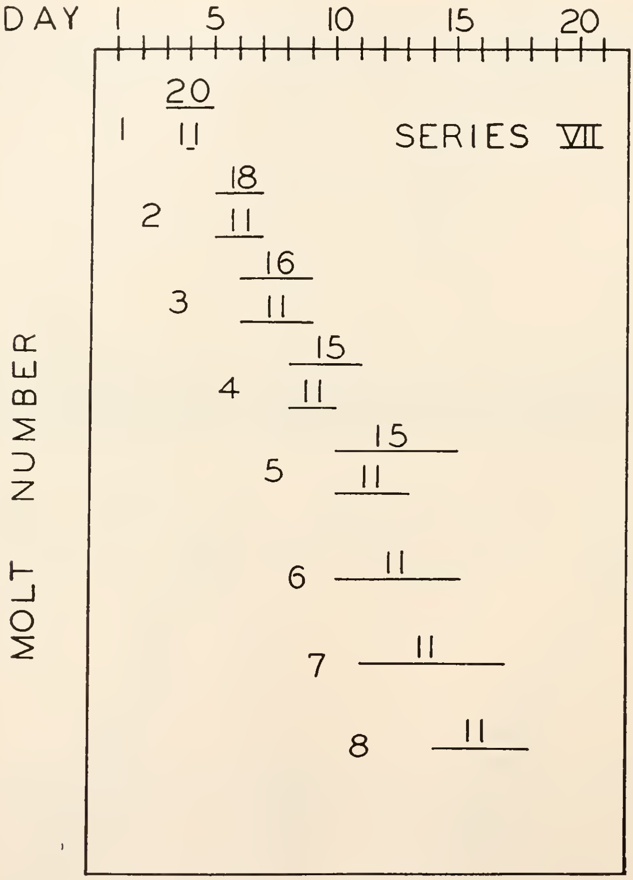

FIGURE 6. Molting periods of *Balanus amphitrite niveus* when reared in twelve hours of light and twelve hours of darkness. Series VII. The top line indicates the number of molts for the entire series. The bottom line indicates the number of molts for those individuals which lived throughout the entire period.

adequate food. Therefore, it must be concluded from the evidence at hand that the first molt in *B. amphitrite niveus* does not occur until the third–fifth day, the majority on day 4.

Growth

Series II, III, IV, and VII were analyzed for molting frequency and, in addition, the areal increase of each barnacle was computed daily. From the accumulative daily areal increase, as plotted for Series II and IV, it is noted that the growth rate of Series II, grown in 24 hours of light, was less than that for Series IV, grown in continuous darkness (Fig. 8). One might question why shell growth in those cultured in total darkness is greater than in those reared in continuous light. In both conditions CO_2 is given off by the unicellular algae, but in those with continuous light it is utilized in the photosynthetic process. Since this synthesis is curtailed in total darkness the carbon dioxide would accumulate. Thus it would seem that more carbonate would be available to barnacles reared in total darkness and this may conceivably have a direct relationship to shell growth.

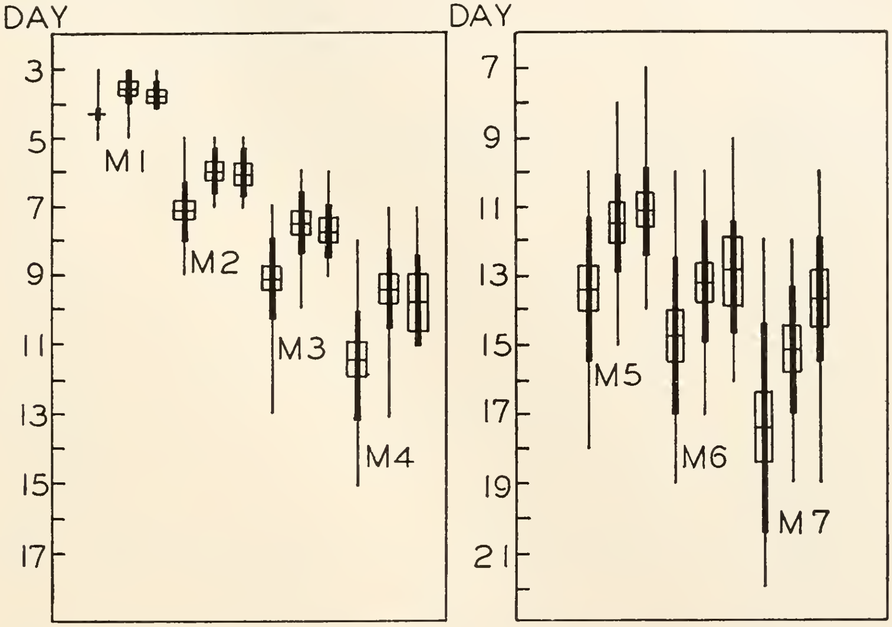

FIGURE 7. Molting frequency of *Balanus amphitrite niveus* under three conditions of light. For every molt a graph is given for each light condition; left line, barnacles exposed to continuous light; middle line, barnacles exposed to continuous darkness; right line, barnacles exposed to twelve hours of light and twelve hours of darkness.

KEY

The vertical line indicates the range.
The solid line indicates one standard deviation on each side of the mean.
The hollow rectangle indicates twice the standard error on each side of the mean.
The cross bar indicates the mean.

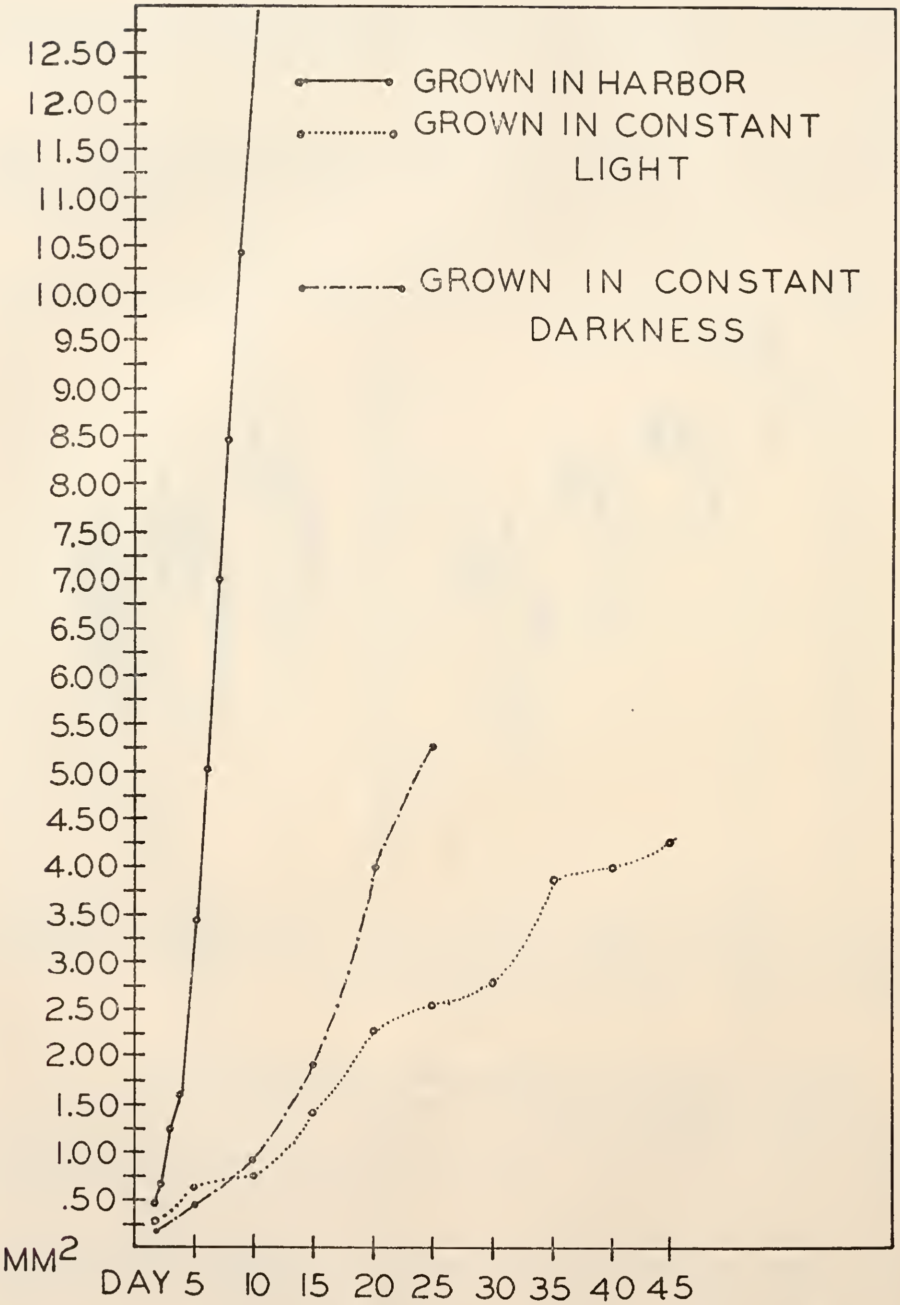

FIGURE 8. Comparison of daily increase in areal diameter in *Balanus amphitrite niveus* reared in continuous light (Series II), continuous darkness (Series IV), and in the natural environment of the harbor (Series VIII).

Periodic fluctuations in shell growth rate were evident in the series grown in continuous light, but not in the series grown in total darkness (Fig. 8), even though they were maintained on the same diet in a constant temperature cabinet. These fluctuations were not evident in *B. improvisus* and the cause in *B. amphitrite niveus* is not known. Unfortunately, Series VII, reared in twelve hours of light and twelve hours of darkness, and the second series grown in continuous light, Series III, could not be maintained beyond 18 days. Thus sufficient data are not available to allow comparisons between these series and the others. Growth in the laboratory, under the conditions described, was never as rapid as that observed for barnacles grown in the harbor.

Mortality

In spite of the uniform conditions provided by a culture cabinet, mortality continued to be very high, even though the barnacles were provided with the same diet which furnished the best growth in *B. improvisus* with practically no mortality (0.5%). The question arose as to whether death could be due to intrinsic conditions, a particular time in the life cycle, or a specific period within the molting cycle. Examination of Figure 9 indicates that the greater percentage of mortality occurs during the first two molting periods, followed by a steady decrease in succeeding molts. Mortality of *B. amphitrite niveus* in the harbor is also very high, especially during the first week after settling. Records have been kept which show the number of barnacles settled on a 100-cm.^{2} plate over a period of a month. These records include recently settled as well as older individuals for each week of the month. An analysis of plates from June, 1953, indicates that at 5 ft. below mean low water 18 of an original 84 pinheads remained alive after the first week (78.5% mortality). At 6 ft. below mean low water 13 out of 51 survived the first week (74.5% mortality). A portion of this was apparently due to predation since fragments of the whole animal remained attached to the tile after the first week.

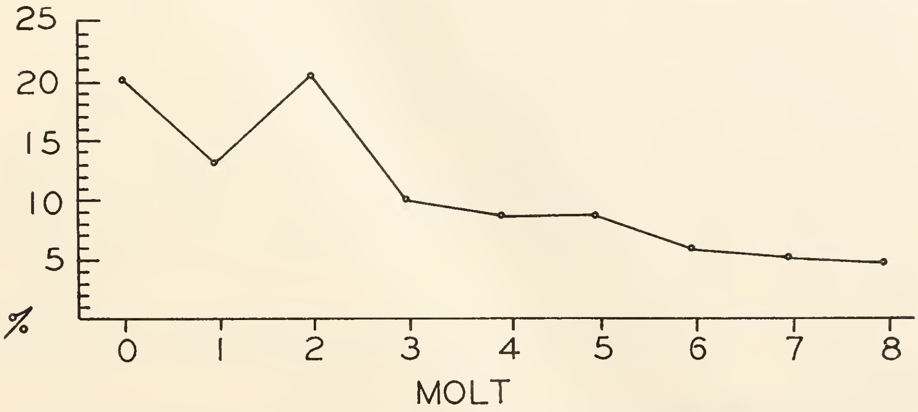

FIGURE 9. Percentage of mortality in relation to molting in *Balanus amphitrite niveus*.

In the analysis of molting and growth separate records were made of each individual which lived throughout the entire period. It was believed that these represented the healthiest barnacles and would more nearly approach those which continue to live in the harbor. An examination of Figures 1–6, giving data for all the barnacles in the individual series as well as those which lived throughout the study, reveals that while the periods for any particular molt are similar for the two groups, the molting periods of the barnacles which continued to live are usually more regular. It is probable that the greater irregularity in the entire series is due to the number of individuals whose metabolism and vigor is reduced and hence, feeding is less than in the more active barnacles. It has been observed that barnacles which molt regularly and live throughout the entire period of study normally cease feeding when disturbed but quickly resume their sweeping activities. Barnacles which show an irregular pattern of molting and shell growth may remain inactive for considerable periods of time after being disturbed.

CONCLUSIONS

From a study of 314 *Balanus amphitrite niveus,* reared in the laboratory, the following conclusions may be made:

1. The first molt occurs from day 3 to day 5, the majority on day 4.
2. The second molt occurs from day 5 to day 9, the majority on day 6.
3. The third molt occurs from day 6 to day 13, the majority on day 8.
4. The fourth molt occurs from day 7 to day 15, the majority on day 10.
5. The fifth molt occurs from day 8 to day 18, the majority on day 12.
6. From the 6th to the 19th molt there is little uniformity in the time of any specific molt, resulting in a considerable spread over which one particular molt may occur. This resembles the condition in *Balanus improvisus* after the 9th molt.
7. *Balanus amphitrite niveus* may pass through 19 molts in a period of 47 days after the time of settling.
8. The average intermolt period for *Balanus amphitrite niveus* is 2–3 days.
9. Statistical analysis shows that there is no significant difference in molting frequency in those series reared in 24 hours of darkness, 24 hours of light, or in those reared in 12 hours of light and 12 hours of darkness.
10. Accumulative daily areal increase of basis in a series of barnacles grown in total darkness showed a greater growth rate than the series reared in continuous light.
11. No association between the molting cycle and shell growth, as determined by daily increase in area of basis, was found.
12. *Balanus amphitrite niveus* differs from *Balanus improvisus* in that it will not pass through the first few molts unless adequate food is available.
13. The greatest percentage of mortality in *Balanus amphitrite niveus,* reared in the laboratory, occurs during the first two molting periods and is followed by a steady decrease in mortality through successive molts. In the natural environment of the harbor the highest mortality is found during the first week after settling which corresponds to the condition observed in the laboratory.

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SPAWNING AND EGG PRODUCTION OF OYSTERS AND CLAMS

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Many aspects of the physiology of reproduction of oysters and clams have been investigated, and most investigators have been impressed by the great number of eggs produced by these animals. Although the number of eggs released, by individual females during a season, is an important factor in determining what constitutes a sufficient breeding stock, this number had never been determined experimentally, and estimates of the number have varied widely.

Brooks (1880) was the first to estimate the number of eggs produced by a female American oyster, *Crassostrea virginica*. His estimate was based on the volume of eggs washed out of ripe females and the calculated volume of a single egg. From the volume calculation Brooks' figures gave 18,750,000 to 125,000,000 eggs, but he believed that as much as 50 per cent should be allowed for foreign matter washed out with the eggs. His estimate, therefore, was nine million eggs per female but states that "an unusually large oyster" gave an estimated 60 million eggs. Churchill (1920) estimated over 16 million eggs and Nelson (1921) estimated 16 to 60 million eggs per female. Galtsoff (1930) observed that individual oysters may release 15 million to 114.8 million eggs at a single spawning and estimated that the maximum number released by a single female during a season may be close to half a billion. Burkenroad (1947), although offering no experimental data, apparently believed Galtsoff's estimate was some ten times too high.

The only known estimate of the number of eggs released by a female clam, *Venus mercenaria,* is that of Belding (1912) who merely states in his summary that the average number of eggs for a 2½-inch quahaug is about two million.

The experiments we are reporting were designed to determine the total number of eggs actually produced by individual oysters and clams under experimental conditions, and to find whether there was any correlation between the size of the individual and the number of eggs produced. We also hoped to find whether this total was affected by the time interval between spawnings, or by the number of times the female spawned.

For our first experiment, 75 oysters were brought into the laboratory on December 30, 1954, and placed in conditioning trays of running sea water at 10.0°–13.0° C. The temperature of the sea water in the trays was raised to 20.0°–21.0° C. on the 31st of December. The clams were obtained on January 5, 1955 and 75 individuals were placed in running sea water at 18.0°–19.0° C. (Loosanoff and Davis, 1950).

In mid-January, after approximately two weeks at conditioning temperature, we started spawning 25 of the clams and 25 of the oysters at three-day intervals. A second group of 25 oysters was spawned at five-day intervals and a third group of 25, at intervals of seven days. The second group of 25 clams was spawned at intervals of seven days and the third group of 25 clams, at intervals of 14 days. To determine the number of eggs produced by each female, each individual was placed in a separate spawning dish, and the shells of the females were marked with a sex symbol and an individual number. The males were discarded as soon as they were identified. The number of eggs released by each female was determined and recorded each time she spawned (Tables I through VI).

The spawning records of the oysters subjected to spawning stimuli every three days are shown in Table I. Within this group the total number of eggs per female ranged from 7.8 million to 59.9 million, while the number released at a single spawning ranged up to 28.3 million. The number of spawnings per female ranged from two to 16. Only number 14 failed to spawn on two or more consecutive three-day trials, and number 10 spawned repeatedly at three-day intervals. The highest number of eggs (59.9 million) was produced by a female that spawned nine times. The female that spawned 16 times ranked second, and a female that spawned only five times ranked third. The lowest total number of eggs was released by a female that spawned seven times. Tables II and III show similar data for the groups spawned at five- and seven-day intervals.

Table I

Number of eggs (in millions) released by individual female oysters induced to spawn at 3-day intervals

Days after initial spawning	Female number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	9.0	2.0	6.8	7.9	6.3	0.4	<0.1	—	—	—	—	—	—	—
3	1.0	0.8	—	3.8	8.7	0.5	2.0	0.9	—	—	—	—	—	—
6	3.1	8.4	—	—	—	—	6.8	2.9	<0.1	<0.1	—	—	—	—
9	—	—	0.4	6.9	16.3	2.5	3.3	1.3	—	0.1	1.8	3.3	—	—
12	2.8	18.7	—	1.6	—	1.1	7.7	2.9	—	4.0	2.7	2.3	—	—
15	—	—	—	—	—	—	—	—	—	9.2	—	—	—	—
18	3.9	5.8	—	4.7	—	1.5	—	2.0	7.1	6.5	—	—	5.6	—
21	—	—	—	—	—	—	9.7	—	—	5.6	—	—	1.8	—
24	3.8	—	—	3.5	—	1.8	—	3.6	—	4.1	28.3	16.5	3.6	—
27	0.9	9.6	—	—	10.1	0.02	7.0	2.3	—	4.0	—	0.1	3.4	—
30	—	—	—	—	—	—	—	—	—	2.8	—	—	—	—
33	4.0	—	—	—	—	—	—	6.6	—	—	—	—	6.5	—
36	—	—	—	—	—	—	—	—	—	5.9	—	—	—	—
39	1.0	—	1.4	—	—	—	3.5	—	—	3.6	—	—	3.5	—
42	—	9.4	0.5	3.7	—	—	—	—	—	2.0	1.2	—	1.7	8.4
45	0.3	—	—	—	—	—	—	—	27.3	2.4	—	—	1.4	—
48	—	—	—	—	—	—	—	—	—	—	—	—	—	—
51	—	5.2	—	—	—	—	—	3.2	—	2.7	9.1	—	1.5	—
54	—	—	—	—	—	—	—	—	—	0.6	—	—	—	—
57	—	—	—	—	—	—	—	—	—	0.2	—	—	—	—
60	—	—	—	—	—	—	—	—	—	—	—	—	—	—
63	—	0.03	1.9	—	—	—	—	—	—	—	—	—	—	2.1
66	—	—	<0.1	—	—	—	—	—	—	—	—	—	—	—
Total eggs	29.8	59.9	11.0	32.1	41.4	7.8	40.0	25.7	34.4	53.7	43.1	22.2	29.0	10.5

The record for clams spawned every three days (Table IV) shows a range from 17.1 to 37.4 million in the total number of eggs released. The highest number released by any female in this group at a single spawning was 17.7 million. The num-

TABLE II

Number of eggs (in millions) released by individual oysters induced to spawn at 5-day intervals

Days after initial spawning	Female number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	0.7	0.7	—	—	—	—	—	—	—	—	—	—	—	—
5	—	5.2	0.2	—	—	—	—	—	—	—	—	—	—	—
10	3.4	—	1.2	1.6	1.0	6.8	7.0	3.4	0.1	—	—	—	—	—
15	—	—	5.9	1.1	2.9	—	—	5.5	9.5	—	—	—	—	—
20	—	—	—	3.1	—	—	—	8.4	—	—	—	—	—	—
25	10.0	—	11.4	—	5.4	—	—	—	10.4	10.1	7.0	—	—	—
30	11.8	32.8	10.6	9.1	1.9	15.5	12.6	8.4	6.6	—	—	2.6	35.6	9.3
35	—	—	—	3.0	0.6	—	—	1.7	—	9.2	—	—	—	—
40	7.4	—	16.1	1.8	0.1	—	—	—	—	—	—	—	—	—
45	1.5	8.9	—	—	—	—	0.7	—	1.2	—	—	—	11.0	—
50	—	—	1.2	—	—	—	—	0.3	—	4.3	—	—	—	—
55	6.0	4.2	—	—	—	10.7	—	—	0.3	—	—	9.1	—	10.8
60	—	—	—	—	—	2.3	—	—	0.2	—	—	0.8	—	—
65	0.9	0.4	<0.1	—	—	—	—	—	—	0.2	—	<0.1	—	—
70	0.2	<0.1	—	—	—	—	—	—	—	—	—	—	—	—
75	0.1	—	—	—	—	—	—	—	—	—	—	—	—	—
Total eggs	42.0	52.2	46.6	19.7	11.9	35.3	20.3	27.7	28.3	23.8	7.0	12.5	46.6	20.1

ber of spawnings per female ranged from three to 10, and the female producing the highest total number of eggs spawned seven times. Second highest total was by a female spawning nine times, and the third ranking female spawned only three times. The lowest total was by a female that spawned five times. Tables V and VI give similar data for the clams spawned at seven-day and at 14-day intervals.

The three groups of oysters, spawned at different intervals, were compared after arranging the females according to their rank in egg production (Table VII). The range in total number of eggs per female was from 10 thousand to 66.4 million.

TABLE III

Number of eggs (in millions) released by individual oysters induced to spawn at 7-day intervals

Days after initial spawning	Female number														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	0.9	0.8	0.2	—	—	—	—	—	—	—	—	—	—	—	—
7	0.8	0.4	—	12.6	—	—	—	—	—	—	—	—	—	—	—
14	21.0	22.2	5.4	10.1	22.0	0.5	0.3	0.2	0.7	0.4	—	—	—	—	—
21	—	—	—	—	—	—	3.1	—	—	1.0	1.4	7.7	0.4	—	—
28	19.4	39.0	—	—	5.7	—	—	23.4	8.6	—	14.2	—	—	—	—
35	7.7	1.6	10.3	—	—	—	17.0	—	2.5	8.4	25.2	—	9.0	0.5	—
42	1.8	1.9	0.9	4.0	—	48.8	—	—	—	0.7	6.2	12.2	1.4	—	0.01
49	0.6	—	—	<0.1	—	—	—	13.8	—	—	—	—	—	—	—
56	—	0.5	—	—	0.6	3.3	—	—	—	—	3.2	—	—	—	—
63	<0.1	—	—	—	<0.1	—	—	—	—	—	0.1	—	—	—	—
70	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total eggs	52.2	66.4	16.8	26.7	28.3	52.6	20.4	37.4	11.8	10.5	50.3	19.9	10.8	0.5	0.01

TABLE IV

Number of eggs (in millions) released by individual clams induced to spawn at 3-day intervals

Days after initial spawning	Female number										
	1	2	3	4	5	6	7	8	9	10	11
0	0.3	7.7	1.1	9.3	4.9	8.5	13.6	5.7	4.7	—	—
3	—	—	—	—	—	—	—	—	—	—	—
6	—	2.1	—	—	—	—	6.4	—	—	13.5	—
9	6.4	—	—	3.5	5.9	3.9	—	—	5.4	1.3	11.1
12	—	—	—	—	—	—	—	—	—	—	—
15	2.1	4.2	17.7	1.6	2.5	2.6	4.0	6.3	1.6	2.3	2.5
18	1.4	—	—	—	—	—	—	—	1.0	—	—
21	0.7	3.5	—	—	4.0	0.1	—	1.9	—	3.1	—
24	1.7	—	10.0	2.4	—	3.4	—	0.7	—	1.1	3.7
27	1.1	—	—	1.0	2.2	1.8	6.3	—	—	—	1.8
30	1.4	—	—	—	—	—	—	—	—	—	—
33	—	—	—	—	1.8	—	—	—	—	1.4	—
36	—	4.2	—	—	—	5.0	—	—	5.0	0.8	1.0
39	—	—	—	—	—	—	—	2.5	—	—	—
42	—	—	—	3.2	3.7	2.2	0.3	—	—	—	—
45	—	—	—	—	0.5	—	—	—	—	—	—
48	—	—	—	—	—	—	—	—	—	—	—
51	3.4	—	—	—	—	—	—	—	—	—	—
54	—	—	—	—	2.4	—	6.6	—	—	—	—
57	—	—	—	—	—	—	—	—	—	—	—
60	—	—	—	—	0.2	1.9	—	—	—	—	—
63	—	—	—	—	—	—	—	—	—	—	—
66	—	—	—	—	—	—	—	—	0.2	—	—
69	—	—	—	—	—	—	0.2	—	—	1.5	—
Total eggs	18.5	21.7	28.8	21.0	28.1	29.4	37.4	17.1	17.9	25.0	20.1

TABLE V

Number of eggs (in millions) released by individual clams induced to spawn at 7-day intervals

Days after initial spawning	Female number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
0	4.1	2.0	3.5	2.8	0.1	—	—	—	—	—	—	—	—
7	—	—	5.2	—	—	10.2	—	—	—	—	—	—	—
14	3.4	17.8	2.7	4.2	—	2.4	—	—	—	—	—	—	—
21	3.2	0.5	0.2	4.4	19.3	3.4	11.4	13.1	14.2	2.4	5.5	—	—
28	—	5.7	7.2	1.5	—	—	—	2.4	—	—	3.7	12.5	—
35	3.1	0.7	0.7	2.7	10.4	0.4	5.6	1.7	—	2.1	—	—	14.7
42	2.0	4.4	1.6	3.0	—	—	0.2	—	—	—	—	—	—
49	2.1	1.7	1.9	1.6	—	5.2	3.6	3.2	18.8	—	11.1	7.5	4.1
56	1.4	1.5	—	1.4	—	0.3	0.7	0.8	—	3.3	1.6	—	—
63	0.8	0.9	—	0.7	—	—	0.6	0.3	—	—	—	—	1.9
70	0.6	0.4	1.0	—	4.8	—	0.1	0.2	—	0.2	1.5	—	0.2
77	—	—	—	—	2.3	—	<0.1	—	—	<0.1	—	—	—
84	0.8	0.1	0.2	0.6	2.3	—	—	—	—	—	—	—	—
91	—	—	—	—	0.3	—	—	—	—	—	—	—	—
98	0.3	—	—	—	—	—	—	—	—	—	—	—	—
Total eggs	21.8	35.7	24.2	22.9	39.5	21.9	22.2	21.7	33.0	8.0	23.4	20.0	20.9

TABLE VI

Number of eggs (in millions) released by individual clams induced to spawn at 14-day intervals

Days after initial spawning	Female number													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	7.0	—	—	—	—	—	—	—	—	—	—	—	—	—
14	2.0	2.8	21.3	10.0	3.3	5.5	20.4	10.5	6.4	2.7	—	—	—	—
28	7.7	7.3	3.3	3.5	22.4	8.0	8.8	8.8	13.3	18.5	19.8	24.3	10.5	0.7
42	3.2	2.7	3.3	5.4	4.2	4.0	4.3	3.2	4.3	1.1	—	2.7	—	8.7
56	1.4	0.5	2.6	1.6	2.2	1.6	1.3	2.0	1.5	8.0	9.3	9.1	3.4	1.5
70	0.2	—	0.4	0.3	1.1	—	1.6	1.2	0.4	2.9	0.7	—	—	0.1
84	—	—	0.3	<0.1	0.3	—	—	0.6	0.3	1.6	—	—	—	0.2
98	0.1	—	—	—	—	0.2	0.1	0.2	—	—	—	<0.1	1.0	—
112	—	—	—	—	—	—	—	—	—	0.5	—	—	—	—
Total eggs	21.6	13.3	31.2	20.8	33.5	19.3	36.5	26.5	26.2	35.3	29.8	36.1	14.9	11.2

Despite the relatively great differences between females, however, the range in all three groups is essentially similar, and the mean number of eggs per female in all three groups is approximately the same. The 14th and 15th ranking females, spawned at seven-day intervals, appear to have abnormally low totals. Except that

TABLE VII

Total number of eggs (in millions) discharged by individual oysters spawned at 3-, 5-, or 7-day intervals, and by individual clams spawned at 3-, 7-, or 14-day intervals. In each group the females are ranked according to egg production

Oysters				Clams			
Rank	3-day spawning interval	5-day spawning interval	7-day spawning interval	Rank	3-day spawning interval	7-day spawning interval	14-day spawning interval
1	59.9	52.2	66.4	1	37.3	39.5	36.5
2	53.6	46.6	52.6	2	29.5	35.7	36.1
3	43.1	46.6	52.2	3	28.8	33.0	35.3
4	41.3	42.0	50.3	4	28.2	24.2	33.5
5	40.0	35.3	37.4	5	25.1	23.4	31.2
6	34.4	28.3	28.3	6	21.7	22.9	29.8
7	32.2	27.7	26.7	7	21.0	22.2	26.5
8	29.8	23.8	20.4	8	20.2	21.9	26.2
9	29.0	20.3	19.9	9	18.7	21.8	21.6
10	25.6	20.1	16.8	10	18.0	21.7	20.8
11	22.2	19.7	11.8	11	17.0	20.9	19.3
12	11.0	12.5	10.8	12	—	20.0	14.9
13	10.4	11.9	10.5	13	—	8.0	13.3
14	7.8	7.0	.5	14	—	—	11.2
15	—	—	.01	15	—	—	—
Mean	31.4	28.1	27.0	Mean	24.1	24.2	25.4
Average per spawning	4.4	5.5	7.2	Average per spawning	3.6	3.6	4.7
Maximum number of eggs from one female at a single spawning 48,780,000				Maximum number of eggs from one female at a single spawning 24,300,000			

they were the last to spawn, spawned only once, and released exceptionally few eggs, there seems to be no reason to exclude them.

A similar comparison of the three groups of clams spawned at different intervals (Table VII) shows that they have even more closely comparable ranges, in the total number of eggs per female, than do oysters. The mean numbers of eggs per individual in the three groups of clams are also in closer agreement.

An analysis of variance test confirms the conclusion that varying the spawning interval from three to seven days for oysters and from three to 14 days for clams did not affect the total egg production. We believe that the similarity of variances and the agreement of means further indicate that each group represented an adequate sample of the populations used.

From Table VII we see that the maximum number of eggs released by one female at a single spawning, in either clams or oysters, may be greater than the seasonal total of many other females in the same population. We also note that the average number of eggs per spawning increases progressively, in oysters, as the interval between spawnings is increased. In clams there is no difference between the groups spawned at three- and seven-day intervals, but the average number of eggs per spawning increases slightly as the spawning interval is increased to 14 days.

If we compare clams with oysters, we find that clams are more uniform than oysters in egg production. The average number of eggs per female varied less from group to group (Table VII), and the range in total number of eggs released by different female clams was not so great. Moreover, as shown by the frequency distribution, the number of female clams producing different total numbers of eggs had a fairly normal distribution with a well-defined modal class at 20 to 24.9 million eggs per female, and the arithmetic mean number of eggs per female (24.6) falls within this class. In oysters, by contrast, the distribution was not obviously a normal one, and there is no well-defined modal class near the arithmetic mean (28.8 million). A comparison of the frequencies of individual spawnings in which different numbers of eggs were released also illustrates the greater variability of oysters since, again, the clams have a lesser range and a somewhat better-defined modal class.

The average number of spawnings per oyster decreases progressively as the interval between spawnings increases, while with clams there is little difference among the three groups. An analysis of variance confirms the conclusion that varying the spawning interval for oysters significantly affects the number of times a female oyster will spawn, with those subjected to spawning stimuli at shorter intervals spawning more frequently. The analysis also shows that varying the spawning interval from three to 14 days had no significant effect on the average number of spawnings per individual clam.

To find whether any correlation existed between the size of the female oyster and the number of eggs produced, we chose the volume of the shell cavity as our best criterion of size. The total number of eggs produced, expressed in millions, was then plotted against the volume of the shell cavity in ml. for each female (Fig. 1). In plotting, different symbols were used to differentiate the groups spawned at different intervals. The plot shows that, while the three separate correlations would differ, there is no striking reversal of trend. The data were therefore combined, and one over-all correlation was computed for all oysters in the experiment considered as a single group. The resulting correlation was reasonably good: *r* was .54 (significant at the .01 level for 41 degrees of freedom), which means that about 30 per cent of the variation in total egg production for oysters could be attributed to the differences in size as denoted by cavity volume.

For clams a similar plot of the number of eggs in millions was made against the shell cavity volume in ml. (Fig. 2). Again, we used different symbols for the groups spawned at different intervals, but the data were combined for computing an over-all correlation. For clams, *r* was .38 (significant at the .05 level for 36 degrees of freedom), which means that about 15 per cent of the variation in total egg production could be attributed to the differences in size of the female clams used in the experiment.

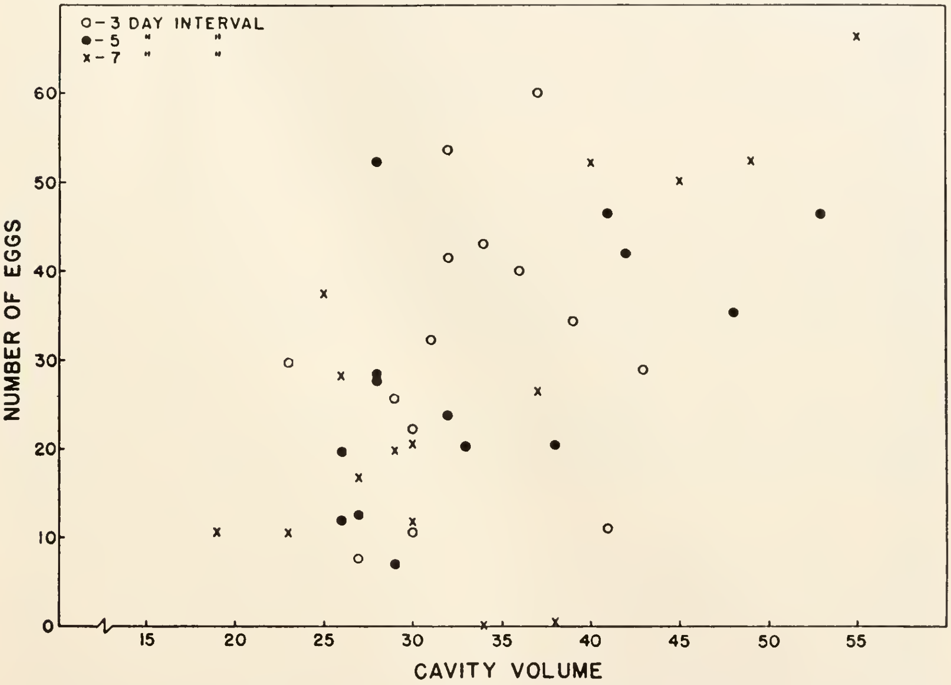

FIGURE 1. Scatter diagram showing the total number (in millions) of eggs produced, plotted against the volume (in milliliters) of the shell cavity, for each of 43 oysters. Different symbols are used to differentiate between females spawned at 3-, 5-, and 7-day intervals.

A similar test was made for a correlation between the number of times a female spawned and the total number of eggs produced. The correlation for oysters was .51 (significant at the .05 level). Thus, in general, females that have a large number of eggs to release will spawn at more frequent intervals than females having a lesser total number of eggs. For clams this correlation was only .17, or was not significantly different from zero.

It should be remembered that the oysters and clams used in this experiment were brought into the laboratory in mid-winter and that conditioning, while in trays of running sea water, does not provide optimum feeding conditions. Field observations in Long Island Sound have shown that, in some seasons, oysters that go through the winter with relatively little glycogen may increase this reserve by as much as two or three times in the spring before gonad development begins. Moreover, Loosanoff and Nomejko (1951) showed that the average gonad thickness of oysters in Long Island Sound, at the beginning of some spawning seasons, may be almost double that recorded at corresponding periods of other years. Although no measurements of gonad thickness or glycogen content were taken at the beginning of the experiment, from inspection we believe that the condition of the oysters used in these experiments was below average for oysters of Long Island Sound.

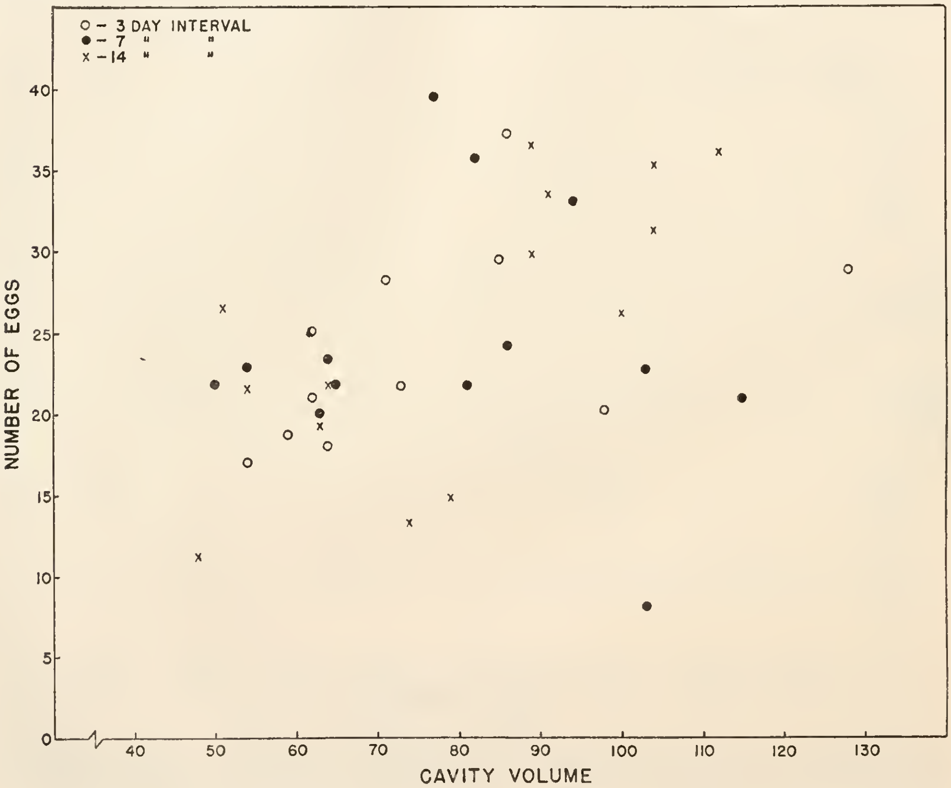

FIGURE 2. Scatter diagram showing the total number (in millions) of eggs produced, plotted against the volume (in milliliters) of the shell cavity, for each of 38 clams. Different symbols are used to differentiate between females spawned at 3-, 7-, and 14-day intervals.

A similar experiment, involving only nine females, was started on June 27, 1955 with oysters that had developed gonads under normal conditions in Long Island Sound. The total number of eggs per female ranged from 23.2 million to 85.8 million, and averaged 54.1 million eggs per female. Thus, both the average number of eggs and the maximum number per female were about 20 million higher than in the winter experiment. This was, we believe, at least in part, the result of these oysters having built up additional reserves of glycogen during the spring prior to the initiation of gonad development. Even these oysters, however, gave far fewer eggs than the ½ billion per female suggested by Galtsoff (1930).

From the present studies, and from another experiment in which 70,000,000 eggs were released by one female at a single spawning, we are in general agreement with Galtsoff (1930) on the maximum number of eggs a female oyster may release at a single spawning. Galtsoff's estimate of ½ billion eggs as about the maximum number a female oyster might release in a single season was based, in part, on the assumption that a female might release approximately 100 million eggs at each of five or six spawnings during a season. Our experiment indicates that this would be unlikely. For example, the female that gave the highest number of eggs at a single spawning, 48.8 million, spawned on only two other occasions during the experiment, and on each of these occasions released less than four million eggs. Of the 43 female oysters in the experiment, only one released in excess of 10 million eggs at as many as three spawnings.

At the conclusion of the experiment, all the females were opened and the gonad condition, presence of shell injuries, and degree of Polydora and sponge infestations were noted for each female. Statistical analysis does not reveal any effect of shell injuries, or of Polydora and sponge infestations, on the number of eggs produced by a female oyster. Histology indicated that only one female oyster still contained a few eggs, but that all the female clams still retained a few apparently mature eggs (Loosanoff, 1937).

We were surprised to find that it required about 2½ months, or approximately the duration of a normal spawning season, to completely spawn out either oysters or clams, even though they were subjected to spawning stimuli every three days. Moreover, there was almost no difference in the time required at the different spawning intervals used in this experiment.

There were marked differences, however, between clams and oysters in their behavior when subjected to chemical and thermal stimulation in the spawning dishes. Usually 80 per cent to 100 per cent of the oysters opened within 15 or 20 minutes after being placed in the spawning dishes, and normally they remained open until after spawning or until they were disturbed. Records available on 183 of the 227 spawnings of female oysters, included in the present experiment, showed how long each female was open before starting to spawn. This period ranged from one minute to 219 minutes, with an average of 34.2 minutes. Approximately 80 per cent of the spawnings occurred 30 minutes or less after the female opened, about 10 per cent of the spawnings occurred within 31 to 60 minutes, and about 10 per cent of the spawnings occurred over an hour after the female had opened and started pumping.

Clams, on the other hand, were much less predictable. A few opened almost immediately after being put in the spawning dishes, some opened only after several hours, and usually there were a few that remained closed throughout an attempted spawning. Even after opening they frequently closed again for variable lengths of time for no apparent reason. The interval between the time the clams first opened and the time they started spawning was recorded for 208 of the 235 spawnings in this experiment. This interval ranged from less than five minutes to 840 minutes, with an average of 137 minutes. Moreover, only 35 per cent of the spawnings occurred within one hour after the clams first opened. Both male and female clams have been observed to spawn for a short time, then, while still remaining open, cease spawning for periods of an hour or more, and finally spawn even more heavily than at first. Also, on several occasions both male and female clams that had been open and pumping vigorously for 30 minutes or more started spawning immediately when placed in dishes of fresh, cooler sea water.

Table VIII

Spawning record of male and female oysters subjected to spawning stimuli daily for five consecutive days; x *indicates spawning*

Females						Males					
Female number	Days					Male number	Days				
	1st	2nd	3rd	4th	5th		1st	2nd	3rd	4th	5th
1	x	—	x	—	—	1	x	x	x	x	x
2	x	—	x	—	—	2	x	x	x	—	x
3	x	x	x	x	—	3	x	x	x	—	x
4	x	—	x	x	x	4	x	x	x	x	—
5	x	x	x	x	x	5	x	x	x	—	x
6	x	—	x	x	x	6	x	x	x	—	—
7	x	—	x	—	x	7	x	x	x	x	x
8	x	x	x	x	x	8	x	x	x	x	x
9	x	—	—	x	—	9	x	x	—	—	x
10	x	—	x	x	—	10	x	x	x	—	x
11	x	x	x	x	x	11	x	x	x	x	x
12	x	x	—	—	x	12	x	x	x	—	x
13	x	x	x	x	—	13	x	x	x	—	x
14	x	—	x	x	—	14	x	x	x	—	x
15	x	x	x	—	—	15	x	x	x	—	x
16	x	x	—	—	—	16	x	x	x	—	x
17	x	x	—	x	—	17	x	x	x	—	x
18	—	x	—	—	x	18	x	x	x	x	x
19	—	x	—	x	—	19	x	x	x	x	x
20	—	—	x	—	x	20	x	x	x	—	x
21	—	—	x	—	—	21	x	x	x	x	x
22	—	—	x	x	—	22	x	x	x	—	x
23	—	—	x	—	—	23	x	x	x	x	x
24	—	—	x	—	—	24	x	x	x	—	x
						25	—	x	x	x	—
						26	—	—	x	—	—

As noted in Table I, only one of the 14 female oysters failed to spawn two or more consecutive times when subjected to spawning stimuli every third day, and female number 10 spawned repeatedly at three-day intervals. This led us to suspect that at least some female oysters might not show the two- to five-day refractory period that Galtsoff found (1938, 1940). An experiment was therefore designed to determine whether some female oysters might spawn at more frequent intervals.

For this experiment, 50 oysters were brought into the laboratory April 4, 1955, and placed in conditioning trays at 20.0°–21.0° C. Twenty-one days later these oysters were placed in individual spawning dishes, and both chemical and thermal stimulation were used to induce spawning. Seventeen females and 24 males responded on the first day (Table VIII). After spawning, the males and females were washed separately in cold running sea water, and the shell of each was marked with the appropriate sex symbol and an individual number. All males were then returned to one conditioning tray, and all the females to another tray. Those oysters that did not spawn on this first trial were returned to a third tray. Each morning, for five consecutive days, all oysters were subjected to spawning stimuli and a record of the spawning of each individual was kept.

As shown in Table VIII, of the 24 females in the experiment, 14 spawned on two or more consecutive days, eight spawned on three or more consecutive days, five spawned on four or more consecutive days, and three spawned on each of the five days of the experiment. As was to be expected, 25 of the 26 males in the experiment spawned on three or more consecutive days, nine spawned on four or more consecutive days, and eight spawned on each of the five days of the experiment. By the fifth day, however, it was difficult to induce spawning and the spawning of both males and females was light. However, when these oysters were again subjected to spawning stimuli, after remaining undisturbed in the conditioning trays for an additional week, 22 of the 26 males and 18 of the 24 females responded, releasing approximately average amounts of spawn.

From our experiments, we believe that either there is no refractory period for female oysters, such as Galtsoff (1938, 1940) described, or it is less than 24 hours in duration. Our results suggest that both male and female oysters can spawn, upon proper stimulation, any time they have physiologically-ripe sex cells to discharge. Under constant, or closely spaced intervals of stimulation, we believe they may so deplete their supply of physiologically-ripe sex cells that they are unable to release more until additional sex cells have become physiologically mature. Some females, for example, were observed to give fairly typical spawning motions on the fourth and fifth days of the above experiment without releasing any eggs and yet, spawned normally after several additional days of conditioning.

We wish to express our thanks to the Director of Milford Laboratory, Dr. V. L. Loosanoff, who suggested this problem, for his advice throughout the experiments, and to our colleagues, Mrs. Barbara Myers, for the statistical treatment, and Mr. C. A. Nomejko, for the figures.

SUMMARY

1. The total number of eggs released by individual female oysters, *C. virginica*, conditioned in mid-winter, ranged from 10 thousand to 66.4 million, and averaged 28.8 million.

2. Females that developed gonads under natural conditions in early summer released from 23.2 to 85.8 million eggs for an average of 54.1 million eggs per female.

3. In this experiment the highest number of eggs released by a female oyster in a single spawning was 48.8 million, but in a previous experiment one female discharged 70.0 million. Thus, some females release more eggs at a single spawning than other females do in a season.

4. There was no significant difference in the average number of eggs released in a season whether the oysters were spawned at three-, five-, or seven-day intervals.

5. Female oysters that had a large number of eggs to release tended to spawn more frequently than females that had a lesser number.

6. The average number of spawnings per female oyster decreased progressively as the interval between spawnings was increased.

7. The total number of eggs produced showed a correlation of .54 (significant at .01 level) with the size of the female oyster, as indicated by shell cavity volume.

8. No correlation could be found between the number of eggs produced and Polydora or sponge infestation, or shell injury.

9. We find no two- to five-day refractory period during which female oysters cannot be induced to spawn, as reported by earlier investigators.

10. The total number of eggs released by individual female clams, *V. mercenaria,* ranged from eight million to 39.5 million, and averaged 24.6 million per clam.

11. The highest number of eggs released by any female clam at a single spawning was 24.3 million.

12. For clams, the correlation between number of eggs produced and volume of shell cavity was .38 (significant at the .05 level).

13. There was no significant difference in the average number of eggs released in a season whether the clams were spawned at three-, seven-, or 14-day intervals, nor was there any significant difference in the average number of spawnings per female.

14. The correlation between the number of times a female clam spawns and the number of eggs produced was not significantly different from zero.

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THE EFFECT OF TEMPERATURE ON TESTICULAR RECRUDESCENCE IN JUNCOS AT DIFFERENT PHOTOPERIODS[1]

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Some, perhaps many, biologists specializing in field observations are unwilling to accept the concept, developed through three decades of experimental work, that length of the daily periods of light and darkness, and their seasonal changes, play a fundamental role in regulating the annual reproductive cycle in birds. Thus, Blanchard (1941, p. 76) deems it ". . . extremely doubtful whether the abundant means which have been discovered for upsetting the physiological balance of captive birds should be accepted as possessing any bearing whatever on the factors which control the cycle under natural conditions." She believes (p. 74) that temperature is the most important single factor lying at the ultimate source of annual variations of the gonad cycle in white-crowned sparrows. Marshall (1951, p. 245) calls for "far more elegant experimental techniques than those used in the past . . . before it can be unquestionably accepted that light fluctuation causes . . . prenuptial sexual recrudescence." He holds (p. 257) that the annual cycle is kept in step with the sun not by the changing photoperiod but by external factors that permit nest building and the events subsequent to nest building, and he includes "mild weather" among the probably most important of these external factors.

The literature dealing with experimental studies of the effect of temperature and day-length on the gonads of birds has been reviewed by Burger (1949). Two papers published prior to that time are especially pertinent to the present study. Burger (1948) found that when starlings are subjected to relatively constant high temperatures (either 32°–35° C. or 38°–40° C.) testicular recrudescence proceeds at a rate faster than when birds are exposed to fluctuating moderate temperatures (11°–24° C., with a daily range of about 6°), providing that the photoperiod is favorable. On the other hand, Kendeigh (1941) found that exposure to fluctuating outdoor temperatures averaging about 2° C. did *not* limit gonad increase under lengthened photoperiod in the English sparrow. More recently Farner and Mewaldt (1952), in a study on white-crowned sparrows of which we have seen only an abstract, conclude (p. 107) that "if photoperiod and light intensity are sufficient to induce early recrudescence, the rate of recrudescence can be accelerated by increased temperature."

The experiments now to be reported do not have the "elegant" design called for by Marshall, but they are the first to test the effect of moderately high and low temperatures on testicular recrudescence in birds exposed to different photoperiods near the supposedly critical or minimum limit. For an understanding of the relative importance of these two factors, temperature and day-length, in the timing of

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the early phases of seasonal reproduction it seems especially important to study their interaction at levels near those occurring in nature at the time these early gonad changes are taking place. The importance of approaching the subject in this way seems obvious, yet in the much-studied field of bird photoperiodism no such study has been made heretofore. The results, we hope, may promote reconciliation of some opposing views and apparently contradictory statements to be found in the literature.

Materials and Methods

Sixty-six male Juncos and two females were used in the experiments. Presumably of the race *Junco hyemalis hyemalis* (Miller, 1941; pp. 321, 329), all were trapped or netted in the immediate vicinity of Chapel Hill, N. C. The birds of groups G, H and J (Table I) were taken in late November and (mostly) in December, 1954; those of groups A through F were captured in January and February (A, B and C in 1954; D, E and F in 1955). Birds collected at any one time were assigned at random to the several groups by the turning of cards. (Scatter diagrams of the results indicate no correlation with date of capture.) Each group of birds was confined in a cage made of ¼ inch-mesh hardware cloth and measuring 36 in. × 24 in. × 24 in.; they were fed a commercial chick mash (Purina Growena). Until experimental lighting was begun (Table I, "Dates"), the birds were held in an unheated attic near a small, north-facing window, hence on nearly natural day-lengths. For the experiments, the cages were placed each in a light-tight, ventilated compartment measuring approximately 60 in. × 48 in. × 48 in., lighted by two 100-watt white-color fluorescent tubes. The lights were operated by automatic timer switches; they delivered approximately 150 foot-candles of light on the floor of the cage. A Taylor maximum-minimum thermometer was placed beside each cage and the temperatures recorded daily. Groups A through F were held on a 10-hour photoperiod until the middle of April; then, for six weeks they were exposed to the test photoperiod (10, 11 or 12 hours), certain of the groups being transferred for this test period to a cold-room maintained at 4°–8° C. Group C was placed in the cold-room during the dark period only, and removed daily to a relatively warm room for the lighted period. At the end of the test period the birds were sacrificed and the testes fixed *in situ* in Helley's fluid. Subsequently, after one week in 80% alcohol, the testes were measured, under a binocular dissecting microscope, with vernier calipers reading to 0.1 mm. From these measurements volumes were calculated according to the rule for a prolate spheroid. Later one testis of each bird was excised and eventually sectioned for determination of spermatogenic state and measurement of tubule diameter.

We chose photoperiods of 12 hours and less because we thought that a longer day-length might mask any temperature effect (as apparently it did in the experiments of Kendeigh, 1941). Also, day-lengths of less than 12 hours more nearly correspond to the natural day-lengths of winter, when birds normally are exposed to lower temperatures. Three different photoperiods were used to permit expression of the effect of length of day independently of the effect of temperature. We chose a test period of only six weeks again to prevent any masking of the effect of temperature—what was to be measured, essentially, was the rate of re-growth of the testes, and over a longer period of time birds exposed to the more adverse experimental environment might have approached the plateau of the growth curve reached earlier by birds enjoying more favorable conditions.

Admittedly, it would have been immensely desirable to begin all test periods on or near the winter solstice, when natural day-length is shortest and when, presumably, the testes of any Junco taken in the field would be in an entirely inactive state. This was not done for the primary reason that we were unable to obtain birds in numbers sufficient to our purpose by that time. Bearing in mind, then, that by February, when some birds in each of the groups A through F were taken, spermatogenic activity had begun, with at least a considerable increase in the number of large Leydig interstitial cells, we held all birds of these groups for about eight weeks on a 10-hour photoperiod, expecting that on such a photoperiod and for such a time all birds would be reasonably "evened up." (We had the further consideration that by late spring the sun would have heated up the attic, where our light-tight compartments are installed, thus increasing the temperature contrast with the cold-room, but this now seems to have been an unnecessary precaution.) At any rate, groups G, H and especially J, which were put on test schedules on Dec. 21, provide some basis for comparison, and for judging the validity of our results.

We are indebted to our colleagues, Dr. Harry Smith and Mr. Edmund Gehan, of the Department of Biostatistics, for statistical analyses of testes volume.

RESULTS

The day before the first three groups were started on a 12-hour photoperiod one bird of group A was found dead, and one also of group B, the deaths presumably due to injury occurring during transfer to clean cages. They provide some indication of the condition of the testes at this time, after about eight weeks on a 10-hour photoperiod (Feb. 16–Apr. 10). The combined volume of right and left testes of one was 2.5 mm.^{3}, the other 1.4 mm.^{3} Both were in stage III (Blanchard, 1941), not yet having reached the stage of division of spermatocytes.

12-hour photoperiod. Six weeks after the beginning of the test period on Apr. 11, the average paired testes volume in the birds of group A had increased approximately 50-fold, reaching 116.2 mm.^{3} (Table I). However, these birds, in warm cages, were far advanced in testicular development over the birds of group B, held in a relatively cold environment, the average volume of the paired testes in group A being approximately four times that of the testes in group B. They were similarly advanced over the birds of group C, which were warmed during the lighted portion of the day but exposed to the cold daily during the dark period. The differences in average volume are significant at the 0.1% level, both between A–B and A–C. The apparent difference between B and C is not statistically significant.

Fully formed spermatozoa were present in at least small numbers in each tubule of seven of the eight birds in group A, but only six of fifteen birds in groups B and C had reached that condition (Blanchard's late stage VI), thus 88% in A, only 40% in B and C. None of the testes from birds of groups B and C had tubules packed with sperm bundles, although four in group A had attained that stage (Blanchard's stage VII).

11-hour photoperiod. The difference between birds exposed to high and low temperatures when on an 11-hr. photoperiod (groups D and E, Table I) is less marked, the difference in average testes volume being significant only between the 5%–10% levels. This low level of significance is largely due to the great variability shown by the birds of group D—in four of the nine, paired testes volume ranged from 18.2 mm^3 to 52.3 mm^3, while in another four it ranged from 2.9 mm^3 to 5.4 mm^3. In group E, however, the largest paired testes volume was 12.2 mm^3; in six of the eight birds it ranged from 3.0 mm^3 to 4.6 mm^3. Of these two groups on an 11-hr. photoperiod the testes of only one bird had developed spermatozoa (Blanchard's late stage VI), this being the most advanced bird in group D. The difference in average testes volume between B (12-hr., cold) and D (11-hr., warm) is not demonstrably significant; that between C (12-hr., warm day, cold night) and D is on the borderline of statistical significance.

TABLE I

Summary of experimental conditions and resultant data

Group	n	Dates	Daily temp. °C.		Photoperiod hrs.	Mean vol. pr. testes mm^3	No. with sperm	Beyond stage III*
			Ave. max.	Ave. min.				
A	8	Feb. 16–Apr. 11 Apr. 11–May 24, '54	‡ 29.0	‡ 23.5	10 12	116.2	7	100%
B	7	Feb. 16–Apr. 11 Apr. 11–May 24, '54	‡ 8.6	‡ 4.0	10 12	26.3	2	100%
C	8	Feb. 16–Apr. 11 Apr. 11–May 24, '54	‡ 25.2	‡ 4.0	10 12	35.6	4	100%
D	9	Feb. 16–Apr. 9 Apr. 9–May 21, '55	26.3 29.9	20.6 24.9	10 11	16.9	1	100%
E	8	Feb. 16–Apr. 9 Apr. 9–May 21, '55	26.6 8.6	20.8 4.5	10 11	5.4	0	75%
F	7	Feb. 16–Apr. 9 Apr. 9–May 21, '55	26.8 29.3	20.6 24.5	10 10	5.2	0	30%
G	8	Dec. 21–Feb. 1, '55	26.3	19.5	11	1.4	0	0
H	7	Dec. 21–Feb. 1, '55	8.2	4.3	11	1.2	0	0
J	4 (+ 2 ♀♀)	Dec. 21–Feb. 16 Feb. 16–Apr. 9 Apr. 9–May 21, '55	25.0 25.9 28.9	19.5 20.6 24.5	11 11 11	14.9	1	100%

* Attainment of stage IV, beginning of spermatocyte division, coincides in white-crowned sparrows with the beginning of territorial activity in resident populations; in migratory populations it shortly precedes start of the spring migration (Blanchard, 1941).

‡ Temperatures were not recorded for groups A, B, and C during the period Feb. 16–Apr. 11, 1954; but these birds were in the same compartments as used for D, E and F in 1955 and temperatures were certainly in the general range 20°–25° C.

When an 11-hr. photoperiod was similarly tested but beginning Dec. 21, advance in spermatogenesis was slight by the end of the test period on Feb. 1 (groups G and H, Table I). The testes of no one of these birds reached stage IV, the beginning of division of spermatocytes, although one in group G was very near to that point. Most were in stage III. The difference between G and H in testes volume is not statistically significant, but it may be noted that it is the cold group H which shows the lowest average volume and that it is the warm group G in which the most advanced individual occurs. Measurements of maximum tubule diameter agree with calculation of testes volume—in the warm group G maximum tubule diameters averaged 75.8 μ, in the cold group H only 66.7 μ.

In another experiment (group J, Table I), four males were exposed to warm temperatures and to a day-length of 11 hours for five calendar months (Dec. 21–May 21). Two females were caged with them throughout this time, an arrangement which we expected would enhance the chances of the males developing larger testes, as has been demonstrated, on a longer day, for starlings (Burger, 1953). In only one Junco of this group, however, did the testes develop to a stage at which spermatozoa were present (45.8 mm^3, late stage VI). The next largest pair of testes had a volume of 6.9 mm^3 (stage V), the smallest only 2.3 mm^3 (early stage IV, *i.e.,* first appearance of spermatocyte division). The range of development was close to that in group D (largest 52.3 mm^3 in D, 45.8 mm^3 in J; 55% under 10.5 mm^3 in D, 75% under 7.0 mm^3 in J).

10-hour photoperiod. For practical reasons having to do with available space and available numbers of male Juncos, a 10-hr. photoperiod was not tested in the cold-room, but only in the warm temperatures of the attic (group F, Table I). None of the seven birds in this test developed spermatozoa, although the testes of one, by far the most advanced, reached a mature stage V, the tubules being packed with primary spermatocytes undergoing division (testes volume 22.6 mm^3). The testes of only one of the remainder showed any dividing spermatocytes (stage IV; testes volume 3.0 mm^3). Testes of five of the seven birds were still in stage III; in three of these, testes volume was less than 2.0 mm^3. The largest pair of testes exceeded the next largest by more than 500%. If this extreme high value is rejected from the statistical analysis, then the difference in the average volumes between group E (11-hr., cold) and group F (10-hr., warm) is significant at the 2%–5% level. That is, in most Juncos recrudescence of the testes proceeds at a measurably faster rate in the cold on an 11-hr. photoperiod than in warmth on a 10-hr. photoperiod.

Discussion

The above results seem clearly to demonstrate that if Juncos are subjected to low temperatures during the period of testicular recrudescence the rate of recrudescence will be retarded (or that, conversely, the rate will be accelerated in birds enjoying a warmer environment). This temperature effect is especially concise in groups A and B, as also in A and C; it is evident, too, in groups D and E. Comparison of groups B and C at least suggests that average daily temperature may be more important than minimum temperature, although the data are insufficient for a precise demonstration.

To this extent our experimental results confirm the conclusions drawn from field observations by Blanchard (1941) and by Marshall (1951) as to the effect of temperature on the annual recrudescence of the avian testis. But additionally they demonstrate, we believe, a decisive limitation on the favorable effects of warm temperature, the limiting factor being a favorable day-length, confirming then also the conclusions of Burger (1948). Two of seven of our birds on a 12-hr. photoperiod in a continuously cold environment developed spermatozoa, as well as four of eight exposed to warm days and cold nights, but only one of nine in continuously warm surroundings on an 11-hr. photoperiod did so. Thus, rate of testicular recrudescence in Juncos under favorable temperature conditions on an 11-hr. photoperiod is certainly no faster, and is perhaps somewhat slower, than it is under poor conditions of temperature but with an additional hour of light (group D versus group B or C). In testes of six of eight birds kept in the cold-room on an 11-hr. photoperiod (group E) primary spermatocytes were undergoing division; on a 10-hr. photoperiod in a warm environment (group F) only two of seven attained this stage. In neither of these groups were spermatozoa developed. If we combine results on different day-lengths (groups A through F) we note that while thirteen of twenty-three birds on a 12-hr. photoperiod (56%) developed spermatozoa, only one of seventeen on an 11-hr. photoperiod (6%) and none of seven on a 10-hr. photoperiod (zero %) did so. Therefore, at these day-lengths and temperatures—both of which are reasonable when compared to conditions in nature—length of the photoperiod is shown clearly to be a more fundamental limiting factor in rate of testicular recrudescence than is temperature. Evidence that this conclusion is valid also for another passerine bird (*Zonotrichia leucophrys gambeli*) is apparent in the data of Farner and Mewaldt (1953), since their individuals kept at about 0° C. on a constant 15-hour day far exceeded, in testicular development, individuals kept in surroundings of 21° C. on natural day-lengths under 12 hours long. Kendeigh's (1941) conclusion that temperatures averaging 2° C. do *not* limit gonad development is not contradictory to our results with low temperatures; the seeming contradiction receives a logical interpretation in terms of day-length. That is, Kendeigh employed a long photoperiod (up to 15 hours), thus further augmenting the already stronger influence of length of day, and this augmentation overrode completely, or at least eventually, the retarding effect of low temperature.

If the validity of our results is questioned because of the several weeks through which we held birds on a 10-hr. photoperiod before beginning the test schedules, we may emphasize justifiably our experiment J, despite the small number of birds involved. These four male Juncos were placed on an 11-hr. photoperiod on the day of the winter solstice; although they were kept warm and had the company of two females, only one had developed spermatozoa even after five months. The testes of this one were next to the smallest of any of the fifteen which developed spermatozoa in these experiments and, judging from tubule size, were just out of the stage of predominance of spermatids. The next largest pair of testes in this group J was smaller than any but one of the twenty-three in our 12-hour groups. The range of development was entirely comparable to that in group D, which were on an 11-hr. photoperiod from Apr. 9 to May 21. All of this strongly suggests to us that on an 11-hr. photoperiod under otherwise favorable experimental conditions most Juncos cannot develop spermatozoa even after several months.

The relatively slight testicular development in those birds exposed to an 11-hr. photoperiod for six weeks beginning at the winter solstice (groups G, H) suggests that our later birds (groups A–F) were somehow conditioned to a more rapid, or greater, response as a result of their long experience of a 10-hr. photoperiod following natural day-lengths to Feb. 16. This is in agreement with the findings of Vaugien (1955) on young house sparrows (*Passer domesticus*). His data demonstrate a progressive increase in testicular weights of sparrows exposed to continuous illumination for four weeks beginning at successive intervals from fall to spring. We would expect, then, if all experiments had been started on Dec. 21, a lesser degree of response in each group after six weeks of treatment. But since all the birds of our groups A to F had experienced essentially identical day-lengths prior to mid-April, the nature of the differences in results can be ascribed confidently to differences in the experimental environment during the ensuing six weeks.

We have not conclusively demonstrated the existence of a minimum or critical length of day, since at least some spermatogenic activity occurred even in our 10-hr. group. The possibility remains that on the shortest days there proceeds, albeit very slowly, at least the earliest stages of recrudescence, *i.e.,* development of functional interstitial cells and multiplication of spermatogonia. Our results suggest, however, that for Juncos days longer than 10 hours are necessary at least to pass beyond the first meiotic division, and it may well be that it is at this stage that length of day becomes the critical factor. An essentially similar conclusion is drawn by Miller (1955) with respect to golden-crowned sparrows (*Zonotrichia coronata*), from results of experiments in which birds were placed on a 10-hr. photoperiod beginning at the winter solstice and sampled at intervals during the following spring and summer. He interprets his data as demonstrating an innate tendency to recrudesce, more or less on schedule, but notes that, on a constant 10-hr. day, this innate process falls short of the level of completion of the maturation divisions, reaching a maximum of stage V with subsequent regression to a resting state.

Our experiments were not continued over as long a period as these of Miller. However, one of our Juncos on a 10-hr. photoperiod reached stage IV, and one which reached stage V approached the next stage, the appearance of spermatids. The relatively advanced state of the testes of this one bird may perhaps point to a further conclusion, when considered together with extreme cases in other groups. In each of five of the six groups A through F, one individual was considerably advanced over all others in its group; in the other group (C) two were so advanced. In group A, the largest pair of testes exceeded the next largest by 25%, in group B this value amounted to 60%, (in C to 10%), in D to 80%, in E to 54%, in F to 510% (and in J to 563%!). In group C the average of the two largest pairs of testes exceeds the third largest pair by 63%; the comparable value in A is 43.5%. That such variations are natural phenomena, and not bizarre experimental results, is evident in recorded field observations, for example, those of Blanchard (1941, p. 56 and Fig. 25) on white-crowned sparrows and of Kirschbaum and Ringoen (1936, Table I) on English sparrows. An explanation for these exceptional Juncos, which we believe to be an entirely plausible and reasonable one, lies in the prospect that individuals vary as to the threshold of response to external stimuli to testicular recrudescence, and that for a very few individuals this threshold is relatively low, occurring even perhaps on a 10-hr. photoperiod and manifesting itself at least under favorable temperature conditions after a long period of time.

Our experiments do not touch on the question as to the point in Junco physiology at which either day-length or temperature exerts its primary or immediate effect. As to temperature it has been suggested by Davis and Davis (1954) that increased thyroid activity induced by a cold environment interferes in some way with testicular activity, possibly from an antagonism of the thyrotropic and gonadotropic functions of the pituitary. At any rate, our results with a 12-hr. photoperiod in cold surroundings show that low temperature alone is not a limiting factor, and such experiments as Kendeigh's indicate that on relatively long days the photoperiodic mechanism completely overrides the temperature mechanism. Also, there are many recorded cases of sperm development in males, and production of fertile eggs in females, in birds exposed to freezing temperatures on a long photoperiod (*cf.* Burger, 1949). We must conclude that day-length is in some way an integral part of the mechanism of testicular recrudescence, being either permissive or prohibitive of sperm development above or below approximately 11 hours in Juncos, and that temperature plays at best a modifying role.

Summary

1. The problem of the environmental control of the initiation of seasonal gonad development in birds was studied experimentally by giving attention to the factors of temperature and day-length at levels near those occurring in nature at the time of normal recrudescence.

2. Forty-seven male Juncos, collected at Chapel Hill, N. C., in January and February, were placed, in groups of seven to nine, on a 10-hr. photoperiod in warm cages from mid-February to mid-April. They were then placed for six weeks on photoperiods of either 12 hours or 11 hours, and in one case continued at 10 hours. One of the 12-hr. groups and one of the 11-hr. groups were transferred to a cold-room at 4°–8° C. for this period. Another 12-hr. group was placed in the cold-room for the dark period only, removed during the daily light period. One 12-hr. group, one 11-hr. group and the 10-hr. group were continued in the warm cages, where temperatures ranged from 24° to 29° C.

3. After six weeks, testes of birds in the 12-hr. warm group were about four times as large as testes of birds in either of the 12-hr. cold groups. Testes of birds in the 11-hr. warm group were nearly as large as those in the 12-hr. cold groups and were larger than those in the 11-hr. cold group. Testes of birds in the 11-hr. cold group were significantly larger than those in the 10-hr. warm group.

4. Thirteen of twenty-three birds on a 12-hr. photoperiod (56%) developed spermatozoa; one of seventeen on an 11-hr. photoperiod (6%) and none of seven on a 10-hr. photoperiod (zero %) did so.

5. Four additional males, in warm (20°–29° C.) cages with two females, were exposed to an 11-hr. photoperiod beginning at the winter solstice; after five months only one had developed spermatozoa. Range of testes development in this group was comparable to that in the 11-hr. warm group above.

6. In another experiment fifteen males were placed on an 11-hr. photoperiod beginning at the winter solstice; after six weeks none had reached spermatogenic stage IV. However, in the 10-hr. group above, one reached stage IV, one stage V.

7. From these data, and from consideration of the pertinent literature, it is concluded *a*) that low temperatures retard the rate of testicular recrudescence in Juncos, higher temperatures accelerating this rate; but *b*) that day-length is in some way an integral part of the mechanism of recrudescence, being either permissive or prohibitive of sperm development above or below approximately 11 hours,

temperature having here only a modifying role; and *c*) that although the existence of a critical, minimum length of day is not conclusively demonstrated, it is not contra-indicated, at least as regards the critical stages of completion of maturation divisions.

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AN ENDOCRINE BASIS FOR THE SEXUAL DIFFERENCE IN MELANIN DISPERSION IN UCA PUGILATOR[1]

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The degree of dispersion of the pigment in the melanophores of fiddler crabs, *Uca* spp., is influenced by a variety of factors. Carlson (1935) demonstrated that blanching resulting from melanin concentration occurred in *Uca pugilator* when the eyestalks were removed. Injection of extracts of the eyestalks caused the *Uca* to darken through dispersion of the pigment. Sandeen (1950) showed that the central nervous organs produced a factor that caused melanin dispersion. Fingerman (1956a) found a blood-borne hormone that concentrated the melanin in the chromatophores of *Uca pugilator*.

Light, temperature, and background also influence the state of the pigment (Brown and Sandeen, 1948). The dispersion of the melanin increased with increase in the light intensity. The black pigment tended to concentrate with increase in temperature. An albedo response was also present. The black pigment tended to disperse on a black background and concentrate on a white background.

Daily and tidal rhythms also determine the dispersion of the melanin. Abramowitz (1937) first described the daily rhythm. The *Uca* were dark by day and light by night. Brown and Webb (1949) and Webb (1950) elucidated the mechanism involved in shifting the phases of this rhythm.

Superimposed upon the daily rhythm is a tidal rhythm (Brown, Fingerman, Sandeen and Webb, 1953; Fingerman, 1956b). This tidal rhythm leads to an increased degree of dispersion of the melanin approximately two hours later than the actual time of occurrence of the tides which are spaced 12.4 hours apart. In regions with semidiurnal tides this augmentation occurred only after the low tide, but in regions of diurnal tides this augmentation occurred after both high and low tides.

Examination of a population of fiddler crabs, *Uca pugilator*, revealed that the females were darker than the males. The following investigation was undertaken to analyze the difference in coloration between the males and females.

MATERIALS AND METHODS

The specimens of *Uca pugilator* used in these experiments were collected near the Gulf Coast Research Laboratory at Ocean Springs, Mississippi, in August, 1955. Several thousand adults were collected and transported on the same day to an air conditioned laboratory in New Orleans, Louisiana, where the room temperature was 24° C. The *Uca* were retained in stainless steel aquaria containing sea water changed every second day. They were kept in constant darkness from the evening of the day they were collected until they were used in the experiments.

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To determine the degree of dispersion of the pigment in the melanophores the method of Hogben and Slome (1931) was used. Each *Uca* was placed on the stage of a stereoscopic dissecting microscope and the average stage of the chromatophores on the anteroventral aspect of a walking leg was determined. According to this system, stage 1 represents maximum pigment concentration, stage 5 maximum dispersion, and stages 2, 3, and 4 the intermediate stages.

Experiments and Results

Sexual difference in the degree of pigment dispersion

Fifty males and 50 females were selected from the stock supply at 10:00 A.M. for use in this experiment. Twenty-five of these females were then placed into each of two white enameled pans. Likewise 25 of the males were placed into each of two white enameled pans. These pans were then placed under a constant illumination of 20 ft. c. intensity. From 4:00 P.M. through 8:00 P.M. the average chromatophore stage of 20 *Uca* from each pan was determined at hourly intervals. After the 8:00 P.M. determination the large chela was removed by autotomy from the 25 males in one of the pans. Male *Uca* have one large chela ("fiddle") and one small chela. Female *Uca* have two small, equal-sized chelae. The small chela on the left side of all the females in one pan was removed at the same time. The average chromatophore stage of 20 of the *Uca* in each of the four pans was then determined at hourly intervals from 8:00 A.M. through noon on the following two days.

This experiment was repeated once. The results have been presented in Figure 1 where each point represents the average of 40 *Uca*. As is evident from section A of the first figure, the normal females were darker than the normal males. The females which had lost one chela became slightly darker than the normal females (Fig. 1; B, C). The normal males remained lighter than the normal females. However, the males which had lost the large chela were darker than both the normal males and normal females but not quite as dark as the females which had lost one chela. The average chromatophore indices for the males without the large chela lay between the values determined for the two groups of females. Apparently, the sexual difference in coloration was due to the presence of the large chela on the males.

To illustrate better the effect of removal of the chelae upon the coloration of both sexes, the data were treated in the following fashion. The five average chromatophore indices obtained on each of the three days for each group of *Uca* were summed. This procedure produced 12 sums. Five was then subtracted from each sum because if there had been no pigment dispersion the sum would have been five. These values were a measure of the chromatophore activities for each day. The average activity for the two groups of normal males was 3.1 activity units and for the normal females 9.4 activity units, a three-fold difference (Fig. 1A).

The activities determined for each of the four groups of *Uca* on the last two days of the observations (Fig. 1, B, C) were summed. The sum of the activities of the normal males was then divided into the sum obtained with the males from which the large chela had been removed. The ratio was 2.8. In like manner, the ratio for the normal females to the females with a chela removed was found to be 1.5, almost a two-fold difference between the males and females.

In order to explain the results presented above the volumes of the large chela and of the body minus the large chela of 10 male *Uca* were determined and the average taken. The large chela of a male had a volume of 1.3 ml., the body and the nine remaining appendages had a volume of 1.5 ml. The results for 10 females showed that the body volume was 1.6 ml. and the volume of one of the small chelae was 0.04 ml.

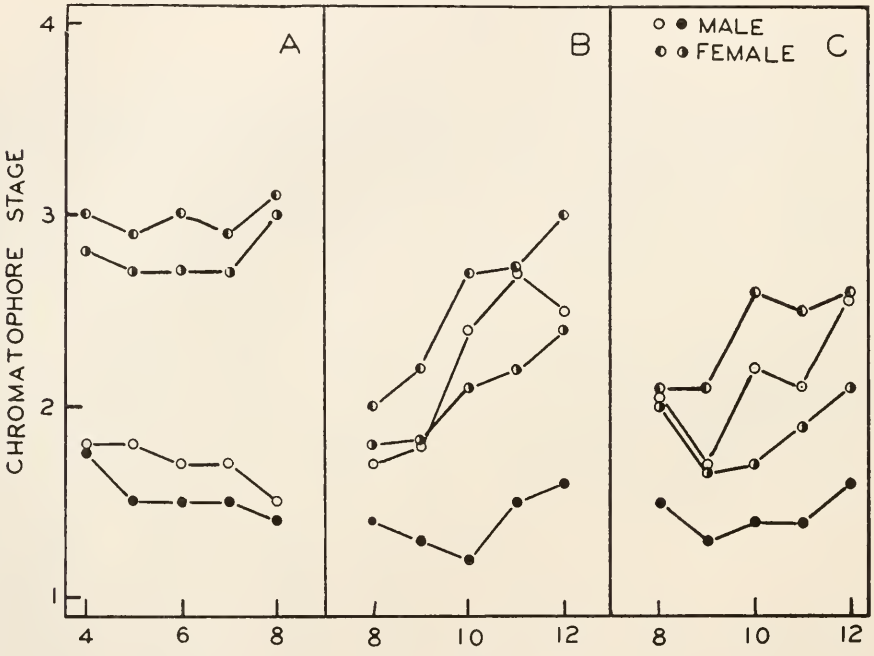

FIGURE 1. A comparison of the degree of pigment dispersion in males and females. In A the males and females were intact, normal *Uca*. In B and C 50 per cent of the males had lost the large chela and 50 per cent of the females had lost one chela.

- ● Males, normal
- ○ Males, large chela missing in B and C
- ◑ Females, normal
- ◐ Females, one chela missing in B and C

Effect of serial removal of appendages

At noon 25 *Uca* were placed into each of nine white enameled pans. Of the 25 *Uca* in each pan approximately 50 per cent were males and 50 per cent of the males had a large chela on the left side. Both sexes were used in this experiment in order to determine the amount of variation that might be due to loss of appendages in experiments where sex is not taken into account. The appendages were arbitrarily numbered consecutively from one to 10 beginning with the chela on the left side. The chela on the right side was appendage number six.

The *Uca* in the first pan were kept intact. The first appendage was removed from the *Uca* in the second pan, the first and second appendages were removed from the *Uca* in the third pan, and so on until the *Uca* in the ninth pan had two appendages remaining. The nine pans were then placed under an illumination of 40 ft. c. intensity. At hourly intervals from 8:00 A.M. through noon on the next two days the average chromatophore stage of 20 of the *Uca* in each pan was determined. The average of the 10 values obtained for each pan was then calculated.

This experiment was repeated once. The only change in the procedure was that the pans were placed under an illumination of 20 ft. c. instead of 40 ft. c. This change had no apparent effect.

The results of both experiments were averaged and have been presented in Figure 2 where each point represents the average of 40 *Uca*. In general, the greater the number of appendages which had been removed the darker were the *Uca*. The curve obtained was sigmoid with the greatest increase in coloration having occurred when seven and eight appendages had been removed.

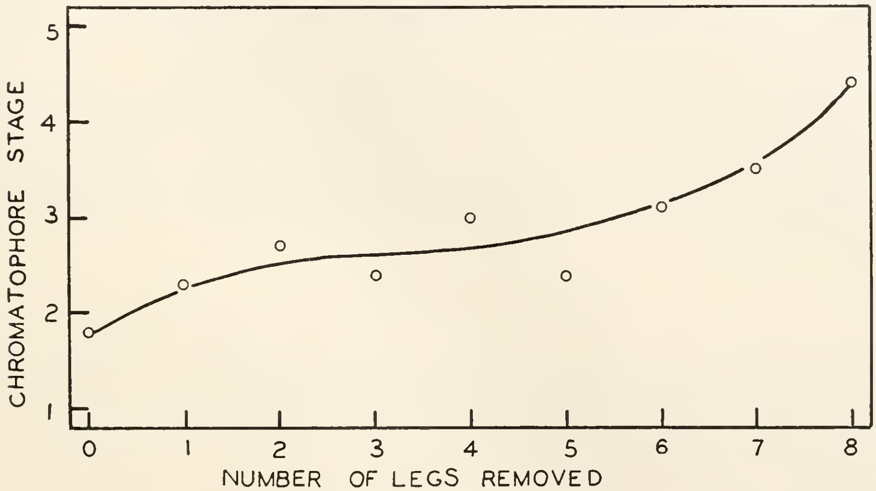

FIGURE 2. A comparison of the degree of melanin dispersion in normal *Uca* with *Uca* from which one to eight appendages had been removed.

To assure that the order in which the appendages had been removed did not effect the results, five appendages were removed from 25 *Uca*, the first three appendages from the left side and the first two from the right side, with no apparent difference in the results. Eight appendages, the first four from both sides, were also removed from 25 *Uca*. The average chromatophore stages of these two groups of *Uca* were similar to the results presented in Figure 2. The order in which the appendages had been removed had no effect upon the results.

DISCUSSION

These experiments have shown that female *Uca pugilator* were darker than the males. The results of the experiments which involved removal of the large male chela showed that this difference in coloration was not a fundamental sexual difference but could be explained merely on the basis of hormonal titers in the blood. This sexual difference in body coloration was due only to the fact that the males with a large chela have a greater circulatory volume than the females. The same quantity of darkening hormone was secreted by the eyestalks and central nervous organs of the male and female *Uca,* but this hormone was diluted to a greater extent in the males which have a greater circulatory volume. Removal of the large chela from the males equalized the difference in the circulatory volume and the males then darkened to the same extent as the females because the hormone was then more concentrated in the blood of the males minus the large chela.

On the basis of the chela and body volumes, the hormonal titer in the blood of males can be assumed to be nearly doubled by removal of the large chela whereas removal of the chela from a female produced a minor volume change. This conclusion, of course, depends upon the assumption that the circulatory volume per unit volume of *Uca* was the same in the body and the large chela.

The second series of experiments also supported this hypothesis. The observation was made that the greater the number of appendages that had been removed, the darker were the *Uca.* This result can also be explained on the basis of a decreased circulatory volume which led to a concentration of the darkening hormone in the blood. An interesting observation is that the *Uca* did not compensate for the loss of the appendages by secreting less darkening hormone.

SUMMARY AND CONCLUSIONS

1. The pigment in the melanophores of female specimens of the fiddler crab, *Uca pugilator,* was more dispersed than the pigment in the males. This difference in pigment dispersion caused the females to appear darker than the males.

2. Removal of the large chela from the male resulted in approximately equal coloration of these males and normal females.

3. This apparent sexual difference in coloration was due to greater dilution of the darkening hormone in the blood of male *Uca* than in female *Uca* because of the greater total volume of the males and was not due to a fundamental sexual difference in the amount of darkening hormone secreted.

4. The greater the number of appendages removed, the darker were the *Uca.* These results have also been explained on the basis of the decrease of circulatory volume in which the hormone had become diluted.

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RESPONSES OF THE WHITE CHROMATOPHORES OF TWO SPECIES OF PRAWNS (PALAEMONETES) TO LIGHT AND TEMPERATURE[1]

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Light and temperature are the major environmental factors governing pigment migration in the chromatophore systems of animals. Light responses have been separated into two categories: (1) responses to total illumination, also known as primary responses, and (2) responses to background, also known as secondary responses. Primary responses are not operative through the eyes whereas secondary responses involve a reflex operating through the eyes. Dispersion of chromatophoric pigments with increased illumination is the typical primary response. Secondary responses typically result in dispersion of dark pigments on a black background and concentration on a white background. White chromatophores usually respond to background in a manner opposite to black pigment. Primary and secondary responses have been demonstrated in several species of crustaceans; among them are *Hippolyte varians,* a shrimp (Keeble and Gamble, 1900; Kleinholz and Welsh, 1937); *Leander serratus,* a prawn (Stephenson, 1934); *Ligia oceanica,* an isopod (H. Smith, 1938); *Uca pugilator,* a fiddler crab (Brown and Sandeen, 1948); and *Callinectes sapidus,* the blue crab (Fingerman, unpublished data).

Background adaptation (secondary response) depends upon the ratio of the light intensity emanating from the light source (incident light) to the intensity of the light reflected from the background. Both incident and reflected light intensities are measured at the surface of the eye. Incident light normally impinges upon the dorsal portion of the compound eye and reflected light upon the ventral portion. The ratio of incident to reflected light intensity is referred to as the albedo.

In 1938, H. Smith demonstrated that background responses of the isopod *Ligia oceanica* were not due simply to the albedo but were complicated by a functional differentiation of the retinal cells or neurons associated with them. By means of experiments in which various portions of the eye were either covered with an opaque material or differentially stimulated by adjustment of the background, he was able to demonstrate that stimulation of the dorsal portion of the eye caused pigment dispersion. Stimulation of the lateroventral portion of the eye caused pigment concentration which resulted in body lightening.

Temperature responses of chromatophores have not been as uniform as light responses. With high temperature, pigments disperse in some species, *e.g.,* the shrimp *Macrobrachium acanthurus* as shown by D. C. Smith (1930) and concentrate in others, *e.g.,* the fiddler crab *Uca pugilator* as shown by Brown and Sandeen (1948).

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Chromatophores of the prawn *Palaemonetes vulgaris* have been the subject of several investigations. Brown (1935a, 1935b) demonstrated that the white chromatophoric pigment of this species disperses broadly when the prawn is on a white background and concentrates strongly when the prawn is on a black background. The white pigment of eyestalkless individuals was found in all states from fully concentrated to fully dispersed with no apparent dependence upon background or light intensity (Brown, 1935a). Hanström (1937) postulated that the white chromatophore of *Palaemonetes vulgaris* is an "independent effector." His experiments suggested that the primary determinant of the state of the pigment was total illumination which caused white pigment dispersion in bright light and concentration in dim light. Responses to total illumination were independent of the nature of the background.

Concentration and dispersion of white pigment of prawns on black and white backgrounds, respectively, are not necessarily due to a specific albedo response. Concentration and dispersion of pigments, especially retinal pigments, in some species may be governed solely by differences in the amount of light reflected from the background with no dependence upon the intensity of the incident light (Sandeen and Brown, 1952; Fingerman, unpublished data).

Neither the physiology of the chromatophores of the prawns *Palaemonetes pugio* and *Palaemonetes paladosus* nor the responses to temperature of the white chromatophores in the genus *Palaemonetes* have been determined previously. The study described herein was undertaken to investigate the white chromatophore responses of two species of prawns, *Palaemonetes pugio* and *Palaemonetes paladosus,* to light and temperature in order to determine the influence of environmental factors upon the white pigment.

Materials and Methods

Specimens of the prawns *Palaemonetes pugio* and *Palaemonetes paladosus* collected near Point Aux Herbes, 15 miles northeast of New Orleans, Lake Pontchartrain, Louisiana, were used in the experiments described herein. Prawns were selected for use without regard to sex or size from stock aquaria which contained diluted sea water at a temperature of 22–24° C. When used in experiments, prawns were placed in pans with a bottom diameter of 14 cm. Each pan contained sufficient water, usually one inch deep, to cover the prawns.

The white chromatophores in the portion of the carapace dorsal to the heart were chosen for observation. Approximately 25 per cent of the individuals of both species did not have white chromatophores dorsal to the heart and were discarded. Males formed a large majority of the discarded specimens.

The white chromatophores were staged with the aid of a stereoscopic dissecting microscope according to the system of Hogben and Slome (1931). Stage 1 represents maximum pigment concentration, stage 5 maximum dispersion, and stages 2, 3, and 4 the intermediate conditions.

Experiments and Results

Responses of white chromatophores to background

Twenty specimens of each species were selected from the stock aquaria and divided into groups of 10. One lot of each species was placed into black enameled pans and the remaining specimens were placed into white enameled pans. The four pans were then placed under an illumination of 40 ft. c. light intensity for 120 minutes after which time the average chromatophore stage of the prawns in each pan was determined. Light intensities were measured by means of a General Electric Photometer. The *Palaemonetes* on the black and white backgrounds were then exchanged so that the prawns which had been originally on a black background were transferred to a white background and vice versa. Average chromatophore stages for the prawns in each pan were then determined 15 and 30 minutes after the backgrounds had been exchanged.

The experiment was repeated once and the results averaged to prepare Figure 1 in which each point represents the average of 20 individuals. As is evident from the figure, the white pigment of both species concentrated when prawns were placed on a black background (dots) and dispersed when prawns were placed on a white background (circles). No species difference in rate of white pigment dispersion or concentration was evident. The white pigment of neither species was maximally dispersed on a white background.

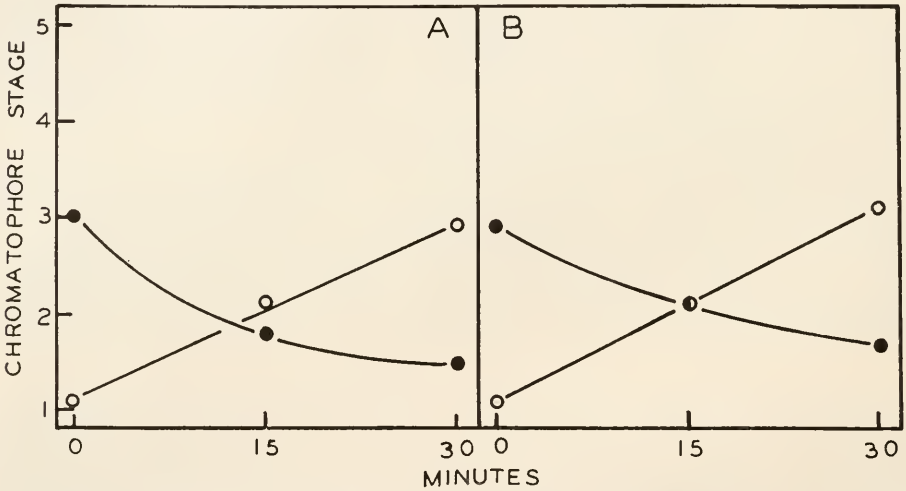

FIGURE 1. Responses of the white chromatophores of *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B) to background changes. Circles, black to white; dots, white to black.

Responses of white chromatophores to illumination

One hundred prawns of each species were selected from the stocks and separated into 10 groups. Five lots of each species were placed in white enameled pans and the remaining groups were placed in black enameled pans. One white and one black pan for each species were then placed under one of the illuminations in the light gradient of 1, 10, 40, 120, and 2000 ft. c. intensity. The latter intensity was obtained with sunlight, the remaining intensities in the light gradient were obtained by placing the pans at appropriate distances from the light sources available in the laboratory. The water in the pans placed in sunlight was changed at frequent intervals to prevent heating of the prawns. After 120 minutes of exposure the average chromatophore index of the prawns in each pan was determined.

The experiment was repeated once and the averaged results are presented in Figure 2 in which the average chromatophore index is plotted against the logarithm of the incident illumination. Each point represents the average of 20 individuals.

The white pigment of both species was more dispersed in prawns on a white background than on a black background at all intensities of illumination. Dispersion of the white pigment of both species of prawn on the black as well as the white background increased with increasing illumination although no significant dispersion of the white pigment occurred in prawns on a black background at illuminations below 120 ft. c. light intensity.

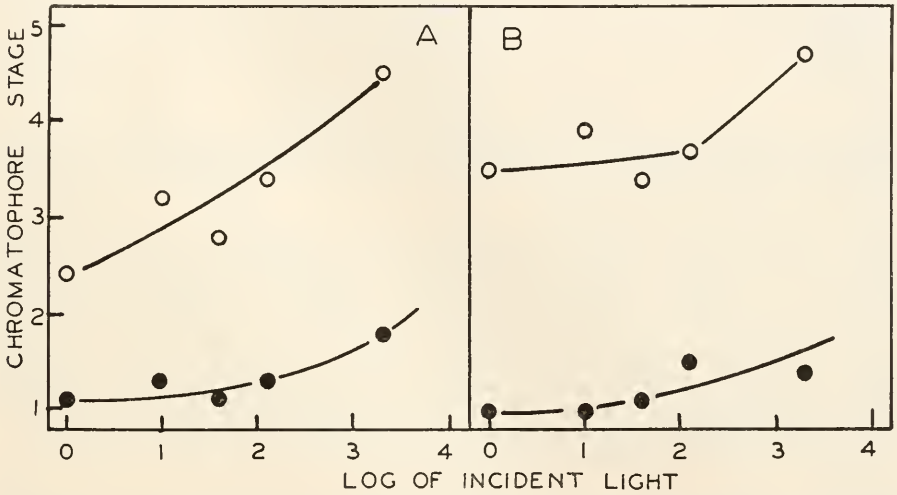

FIGURE 2. Relationship between dispersion of the white chromatophoric pigment of *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B) and the logarithm of the incident light intensity. Dots, black background; circles, white background.

A species difference was evident from the response of the chromatophores of prawns on a white background. The white chromatophores of *Palaemonetes paladosus* showed no response to illumination between the light intensities of one and 120 ft. c. whereas the white pigment of *Palaemonetes pugio* gradually became more dispersed as the intensity of illumination increased from one to 120 ft. c. In addition, the white pigment of *Palaemonetes paladosus* was more dispersed than the white pigment of *Palaemonetes pugio* at all intensities of illumination.

Additional experiments were performed in which prawns whose eyestalks had been removed 24 hours previously were exposed on a white background to the same intensities of illumination and for the same period of time used with the normal prawns. The white pigment of eyestalkless prawns was usually neither completely dispersed nor completely concentrated and frequently could be found in all stages of dispersion in one prawn as was observed in *Palaemonetes vulgaris* by Brown (1935a).

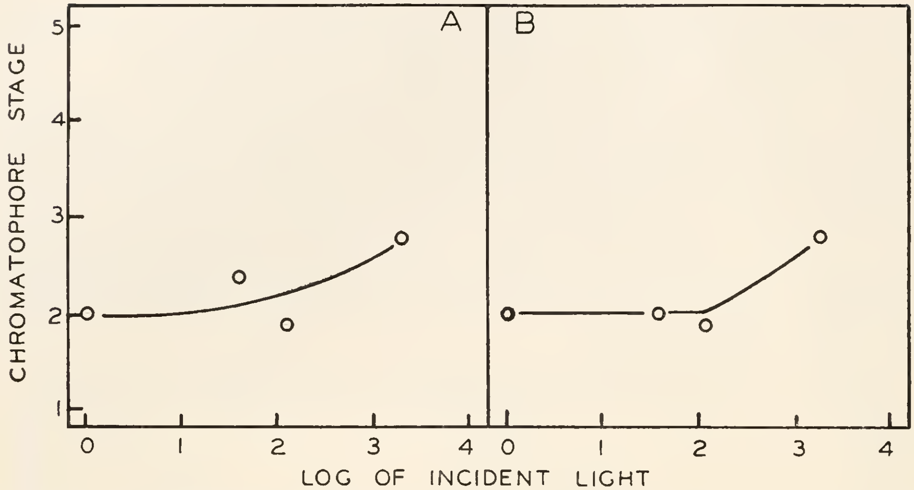

FIGURE 3. Relationship between dispersion of the white chromatophoric pigment of eyestalkless *Palaemonetes pugio* (A) and eyestalkless *Palaemonetes paladosus* (B) and the logarithm of the incident light intensity.

The experiment with eyestalkless prawns also was repeated once. The averaged results are presented in Figure 3 where each point represents the average of 20 individuals. A primary response of the white pigment of both species was evidenced by increased dispersion with increase in the intensity of incident illumination.

In view of the fact that the white pigment shows a primary response, the results presented in Figures 1 and 2 may be explained in terms of differences in the amount of light reflected from the black and white backgrounds that impinges directly upon

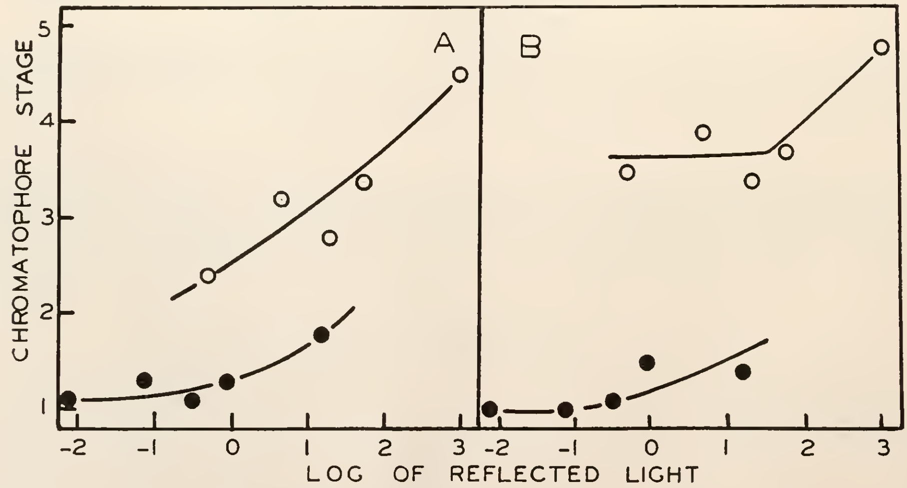

FIGURE 4. Relationship between dispersion of the white chromatophoric pigment of *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B) and the logarithm of the reflected light intensity.

the white chromatophores, rather than as an albedo or background response which is mediated by the eyes. Measurements by means of the General Electric Photometer showed the white backgrounds used in the experiments reflected 50 per cent of the incident light and the black background reflected 0.77 per cent of the incident light, $\frac{1}{65}$ as much light as was reflected from the white background.

The data of Figure 1 were, therefore, re-plotted in terms of the intensity of the light reflected from the black and white backgrounds in order to assist in determining if the albedo is involved in the responses of the white chromatophores on black and white backgrounds (Fig. 4). As is evident from the figure the white pigment of both species responded directly to illumination because dispersion of the white pigment of both species increased with increase of the reflected light intensity. In addition, a specific albedo response was evident because the data obtained on the black and white backgrounds did not overlap although the incident light intensity had increased 2000-fold as compared with only a 65-fold increase in the reflected light intensity.

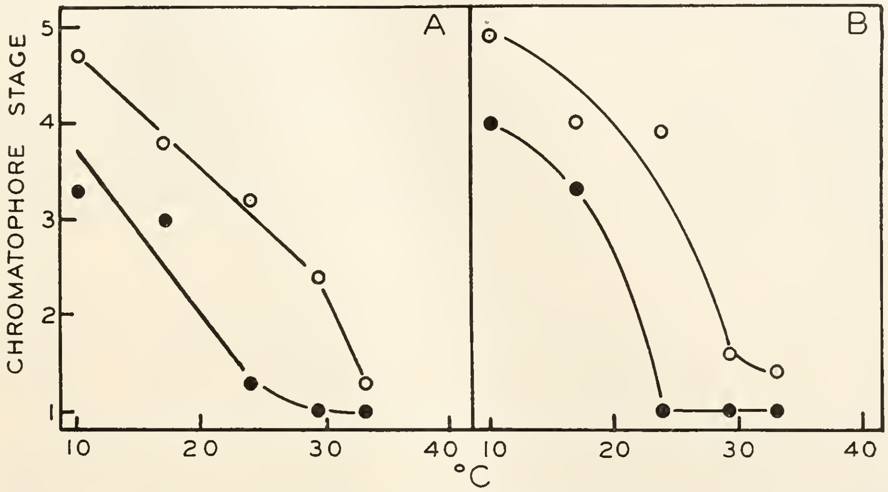

FIGURE 5. Relationship between temperature and white pigment dispersion in *Palaemonetes pugio* (A) and *Palaemonetes paladosus* (B). Dots, black background; circles, white background.

As is evident from Figures 1, 2, 3, and 4 dispersion of the white pigment of both species of *Palaemonetes* is due in part to summation of primary and secondary responses. The response to total illumination is an increased dispersion with increased illumination. The background response results in greater dispersion of the white pigment of prawns on a white background than of prawns on a black background.

Temperature responses of white chromatophores

Ten groups of 10 prawns of each species were selected from the stocks. Five lots of each species were placed in white enameled pans and a similar number in black enameled pans. Sufficient water to cover the prawns was then added and the pans were placed in water baths. The temperatures of the five water baths were maintained at 10, 17, 24, 29, and 33° C., respectively. The incident illumination was 20 ft. c. light intensity. In each water bath were two black and two white pans containing individuals of each species. Each lot of prawns was kept in the bath for 120 minutes after the water in the pans had attained the temperature of the water bath. The average chromatophore index of the 10 prawns in each pan was then determined. The results are presented in Figure 5.

As is evident from the figure, at all temperatures the white pigment was more dispersed on a white background than on a black background. With increase of temperature, the white pigment of individuals of both species became more concentrated on both black and white backgrounds. No difference between the temperature responses of the white pigment of the two species was evident. The white pigment of individuals on a black background became fully concentrated at temperatures at which the white pigment of specimens on a white background was still partially dispersed. The four curves in Figure 5 are as parallel as one could expect in experiments of this sort. One may conclude from this parallel response that the qualitative behavior of the white pigment in response to temperature changes was independent of the nature of the background. Black backgrounds merely decreased the initial dispersion of the white pigment so that complete concentration of the white pigment occurred on a black background at a lower temperature than on a white background.

Discussion

Temperature is equally as important as light in determining the amount of dispersion of the white chromatophoric pigment of the prawns *Palaemonetes pugio* and *Palaemonetes paladosus* under any set of environmental conditions. Maximum white pigment concentration and maximum dispersion were both observed in the experiments which were designed to determine the effects of total illumination and temperature upon the white chromatophores. Background and total illumination both caused maximum white pigment dispersion and concentration in *Palaemonetes vulgaris* (Brown, 1935b; Hanström, 1937). *Palaemonetes paladosus* and *Palaemonetes pugio* are obviously exceptions to the general principle that light is more important than temperature in determining the state of the chromatophore system (Brown, 1950).

A thermoregulatory function of the white pigment does not seem plausible from the data presented herein. If the temperature response of the white chromatophores were thermoregulatory, then with increase in temperature, the white pigment should disperse in order to increase the area of the body surface which is able to reflect light and heat efficiently.

In contrast to the white chromatophores of *Palaemonetes* the melanophores of frogs appear to be thermoregulatory. More light is reflected from the skin of a frog whose black pigment is concentrated because of adaptation to a white background than from the skin of a frog whose melanin is dispersed due to adaptation to a black background (Deanin and Steggerda, 1948). These investigators have also shown that the skin of the frog reflects more red light than violet light, which lends further support to the thermoregulatory function of the frog melanophores because red light has a higher heating ability than violet light.

The white chromatophoric responses of the two species of prawns to temperature are antagonistic to their responses to illumination. With increased temperature the white pigment concentrated, whereas the pigment dispersed with increased total illumination. These antagonistic responses tend to maintain a steady state of the white pigment. Since heat and bright light are concomitant in nature, *e.g.*, sunshine, the tendency of the white pigment to disperse in bright light is counteracted by the tendency of the pigment to concentrate because of the associated heat.

In Lake Pontchartrain, where the *Palaemonetes* were collected, temperature changes probably affect the prawns more than changes in illumination. *Palaemonetes* in their habitat are not normally exposed to bright illumination but the water shows wide variation in temperature.

The white pigment of the fiddler crab *Uca pugilator* responds to temperature differently than the white pigment of *Palaemonetes pugio* and *Palaemonetes paladosus*. The white pigment in *Uca pugilator* tends to disperse as the temperature is increased above, or decreased below about 20° C. (Brown and Sandeen, 1948).

The white chromatophores appear to function as "independent effectors" in response to both total illumination and temperature. Evidence presented herein shows that white pigment concentration and dispersion in *Palaemonetes pugio* and *Palaemonetes paladosus* are due to summation of (1) an albedo response resulting in greater dispersion on a white background, (2) a response to total illumination resulting in greater dispersion in bright illumination, and (3) a response to temperature resulting in greater concentration at higher temperatures. Responses to temperature and total illumination appear to be more important than the albedo responses which are superimposed upon the primary response to total illumination.

Summary and Conclusions

1. Responses to light and temperature of the white chromatophores of two species of prawns, *Palaemonetes pugio* and *Palaemonetes paladosus,* have been investigated.

2. The white pigment is more dispersed in prawns on a white background than in prawns on a black background at all intensities of illumination, due to a specific background (albedo) response.

3. A response to total illumination is evident. The dispersion of the white pigment increased with increase in total illumination.

4. The white pigment became more concentrated with increased temperature in prawns on both black and white backgrounds.

5. The tendency of the white pigment to disperse in bright light is normally antagonized by the tendency of the pigment to concentrate with increased temperature. Since heat and bright light are usually concomitant in nature, as in sunlight, the antagonistic response to light and temperature has been interpreted as a mechanism to maintain a steady state of the white chromatophores.

6. At all intensities of illumination the white pigment of *Palaemonetes paladosus* was more dispersed on a white background than was the white pigment of *Palaemonetes pugio*. In distinction to *Palaemonetes pugio* the white pigment of *Palaemonetes paladosus* on a white background showed no response to illumination between one and 120 ft. c. light intensity.

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ANTIMITOTIC SUBSTANCES FROM THE OVARIES OF VERTEBRATES[1]

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More and more evidence has been accumulated to show that the protoplasmic colloid is in a state of dynamic equilibrium between the factors which tend to cause it to gel or clot and those which tend to prevent or reverse this gelation. For a review of much of this evidence, see Heilbrunn (1956).

Substances which are potent in preventing protoplasmic clotting can be obtained from the ovaries of various animals (Heilbrunn, Chaet, Dunn and Wilson, 1954; Heilbrunn, Wilson and Harding, 1951). The fact that the ovaries of some fishes contain such substances has already been noted. In this paper, additional data will be presented, not only for the ovaries of fishes, but also for the ovaries of other vertebrate animals. These substances which prevent protoplasmic clotting also prevent the mitotic gelation which is a necessary precursor of the mitotic spindle; hence such substances have an antimitotic action.

The methods used and the plan of attack were much the same as in the earlier work. Table I shows the results obtained in the summer of 1953. Ovaries from 8 species of fishes all contained substances which had a strong antimitotic action on the eggs of the worm *Chaetopterus pergamentaceus*. The extracts were prepared by homogenizing the ovaries in sea water which had been made acid by the addition of HCl in sufficient amount to break down the buffers of the sea water. Following homogenization, within a few minutes the extracts were centrifuged (at 16,000 g) until most of the solid material was removed, and the supernatant fluid was then brought to a pH of approximately 8, that is to say, to the pH of sea water. In the case of the ovaries of *Sphaeroides maculatus*, the extracts were made in ordinary sea water rather than in acid sea water.

Two minutes after they had been inseminated, Chaetopterus eggs were introduced into the ovarian extracts, used either full strength, or diluted one-half or one-fourth with sea water. The eggs were kept at a temperature of 21° C. In all cases, the cleavage, if it occurred at all in the extracts, was delayed. Cleavage counts were made after a time interval long enough to insure that the percentage of cleavage was as high as it ever would be. In some instances, a cleavage furrow would start to form, but then it would disappear and the egg would become spherical again. Viscosity determinations were made 30–40 minutes after insemination. At this time, normal eggs show increased protoplasmic viscosity. Earlier work (Heilbrunn and Wilson, 1948) had shown that on our arbitrary scale, the viscosity of the protoplasm rises from 7 to 14. This means that it takes 14 seconds, for a cen-

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trifugal force of approximately 2200 g to move the granules of the protoplasm a sufficient distance so that the egg appears to be divided into zones. At this time, then, there is a gelation of some part of the protoplasm; this we call the mitotic gelation. In the control eggs such a gelation was always present. On the other hand, the protoplasm of the eggs exposed to the ovarian extracts always retained its original fluidity, and in some cases at least became even more fluid. This is indicated by the viscosity values shown in the table (Table I). In making these viscosity determinations, it was often not possible to make enough tests to be certain of the exact value for the viscosity, but in every case it was clear that the viscosity of the treated eggs was much less than that of the control (untreated) eggs centrifuged at the same time. These control eggs showed no indication of zones when they were centrifuged at the same force and for the same time as the treated eggs; whereas the eggs treated with a sufficient concentration of the ovarian extract, showed zones clearly. These treated eggs regularly gave viscosity values of 8 or less.

TABLE I

Effects of extracts of fish ovaries on cleavage of Chaetopterus eggs

Species	Extract no.	Grams ovaries	Ml. sea water	Viscosity 30–40 min. after insem.	% cleavage	Control % cleavage
Sphaeroides maculatus (Bloch and Schneider) puffer	1	72	100	6	0	100
Fundulus heteroclitus (Linnaeus) mummichug	6	10	25	4	0	97
	6	10	50	6	50	97
	6	10	100		46	97
	32	2	10	8	39	98
Opsanus tau (Linnaeus) toadfish	7	30	60	6 or less	0	99
	7	30	120	6 or less	0	99
Tautogolabrus adspersus (Walbaum) cunner	8	3	15	6	9	100
	33	1	9	8 or less	42	98
Tautoga onitis (Linnaeus) tautog	20	12	24	6	46	94
	29	15	30	8	15	100
Stenotomus versicolor (Mitchill) scup	22	2	10	8 or less	35	98
	22	2	20		37	98
	22	2	40		75	98
	31	2	10	6 or less	22	97
Anguilla rostrata (LeSueur) eel	35	6	30	8	49	89
Lophius americanus (Cuvier and Valenciennes) goosefish	4	100	100		0	93
	4	100	200		27	93

In the summer of 1954, the work on fish ovaries was continued. We made extracts from the ovaries of 7 other species of fish, and we also made new extracts from the ovaries of the toadfish (*Opsanus tau* L.). In the 1954 work, for each gram of ovary, two ml. of sea water were used to obtain an extract. In the tests, the extracts were used full strength, half strength (that is to say, diluted with an equal volume of sea water), one-fourth strength, and one-eighth strength. In all cases the pH of the extract was adjusted so that it was approximately the same as that of sea water. The results of these 1954 experiments with fish ovaries are shown in Table II. Because of the fact that in most cases extracts weaker than half strength had but little effect, results obtained with these dilute extracts are not included.

Actually, our extracts always contain a mixture of substances which inhibit clotting and substances which promote clotting. If the extracts are allowed to age, the anti-clotting substances seem to lose their potency before the clotting substances do. Hence in extracts kept for a day or two in the icebox, the anti-clotting effect is reduced. Moreover, in one experiment done on the ovaries of fish (mackerel) bought in a fish market, we found that the extracts caused clotting rather than liquefaction of the protoplasm. In every case, the ovarian extracts prepared from freshly caught fish kept the protoplasm fluid and prevented the mitotic gelation. This was determined by tests of viscosity made 30–40 minutes after insemination.

TABLE II

Additional data on the effect of extracts of fish ovaries on the cleavage of Chaetopterus eggs. The figures show percentage of eggs cleaving

Species	Full strength extract	Half-strength extract	Control
Brevoortia tyrannus (Latrobe) menhaden unripe ovary	10	17	100
Same—ripe ovary	1	4	100
Raja erinacea (Mitchill) skate—unripe ovary	0	48	97
Same—ripe ovary	0	80	97
Poronotus triachanthus (Peck) butterfish	96*	90	100
Opsanus tau (Linnaeus) toadfish	0	25	99
Centropristis striatus (Linnaeus) sea bass	10	81	99
Prionotus carolinus (Linnaeus) sea robin	76	86	97
Pseudopleuronectes americanus (Walbaum) winter flounder	11	96	100

* Cleavage was delayed for well over 6 minutes.

Table III shows the effect of extracts of ovaries from vertebrates other than fishes. These extracts also have a strong antimitotic effect, and in general they also prevent the mitotic gelation. Only one experiment with cow ovaries is listed. As a matter of fact, in attempting to discover how to make more potent extracts, we did many experiments with extracts from cow ovaries. The results obtained will be reported later. Suffice to say here that we always found sea water extracts of cow ovaries to exert a strong antimitotic action. However, in making these extracts we found it advisable to mince the ovaries instead of homogenizing them. Also it was not necessary to acidify the sea water in order to obtain a satisfactory extract.

Our results show that the ovaries of various vertebrate animals all contain antimitotic substances. At the present time, we are trying to obtain these substances

TABLE III

Effect of extracts of the ovaries of various vertebrates on the cleavage of Chaetopterus eggs

Animal	Extract no.	Grams ovaries	Ml. sea water	Viscosity 30–40 min. after insem.	% cleavage	Control % cleavage
Frog (*Rana pipiens*)	18	10	20	3	0	—
Frog (*Rana pipiens*)	18	10	40	—	0	98
Frog (*Rana pipiens*)	28	10	30	2	0	100
Frog (*Rana pipiens*)	28	10	60	—	0	100
Frog (*Rana pipiens*)	28	10	120	—	41	100
Salamander (*Amblystoma punctatum*)	38	0.5	5	6	0	99
Salamander (*Amblystoma punctatum*)	38	0.5	10	—	73	99
Chicken	39	26	52	5	2	98
Rabbit	23	2	10	8+	92*	97
Dog	34	2	10	8	3	94
Pig		25	25	<8	0	99
Lamb		14	15	<8	0	99
Cow		90	90	<8	0	100

* Cleavage was delayed 8 minutes.

in relatively pure form, in the hope that they may be able to cause regression of tumors in mice. Preliminary experiments indicate that our crude extracts are capable of causing such a regression, but the results we have obtained thus far vary greatly, and much more work is needed (Heilbrunn, Lippman and Rutman, 1955).

It must not be thought that the ovary is the only organ that contains antimitotic substances. We have made extracts from liver, testis and muscle, and all of these extracts prevent cell division. This is indeed what we would expect, if as we have said, protoplasm usually, perhaps universally, contains substances which have an inhibiting effect on protoplasmic clotting.

SUMMARY

1. Extracts from the ovaries of 14 different species of fishes all have an antimitotic effect on the eggs of the worm Chaetopterus.

2. Extracts from the ovaries of the frog, salamander, chicken, rabbit, dog, lamb, pig, and cow act in similar fashion.

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THE PHYTOPLANKTON OF GREAT POND, MASSACHUSETTS[1]

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Although the plankton flora of moderately deep estuaries has been studied to some degree (Braarud and Bursa, 1939; Cowles, 1930; Gran and Braarud, 1935; Lohmann, 1908; Morse, 1947) very little quantitative study of phytoplankton in very shallow estuaries has been done. Several investigators in Belgium and Holland (Conrad, 1926a, 1926b; Massart, 1920; Van Goor, 1925a, 1925b, 1925c) have studied taxonomically the phytoplankton of very shoal estuaries. The forms they describe are almost entirely flagellates. It would be of great interest to learn whether these taxonomic studies indicate a distinctly different flora composed primarily of flagellates or whether diatoms, as well as flagellates, thrive in very shallow estuaries. Further, in the event that flagellates should dominate in shoal estuaries, it would be of interest to characterize the conditions associated with their dominance.

THE EMBAYMENT INVESTIGATED

Figure 1 shows the form and depth of Great Pond. A narrow gut admits water from Vineyard Sound at one end and a small river, the Coonamessett, empties at the other end. It is divided into a wider seaward portion, the main pond, and a narrower landward portion, the arm. Its depth is nowhere greater than 9 feet at highest high tide. The hydrography of the Pond has been well studied by Barlow (in press), but a few of the salient features are reviewed here. The mean salinity indicates that the Pond water is 83 parts sea water and 17 parts river water. Surface salinity decreases from 32‰ in the Sound to 1–2‰ near the mouth of the River. Bottom salinity decreases from 32‰ to 24‰ near the mouth of the River, indicating marked stratification in the arm. The effect of wind blowing toward the Sound is to move the surface water seaward, resulting in upwelling of deep water and increase of surface salinities to 24‰ or above all the way to the River.

Water samples were taken at the stations indicated in Figure 1 from the shore in February and March and from a rowboat during the rest of the year. Samples were taken within several inches of the surface and approximately ten inches from the bottom. Phytoplankton cell counts and phosphate, turbidity, and salinity determinations were made on each sample.

METHODS

Cell counts were made from four to forty-eight hours after samples were collected. Cell concentrations were determined by counting living cells in a very shallow chamber, so that a high power microscope objective could be used. Usually the water sample was concentrated on a smooth-surfaced porcelain filter with a pore

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[1] Contribution No. 764 from the Woods Hole Oceanographic Institution.

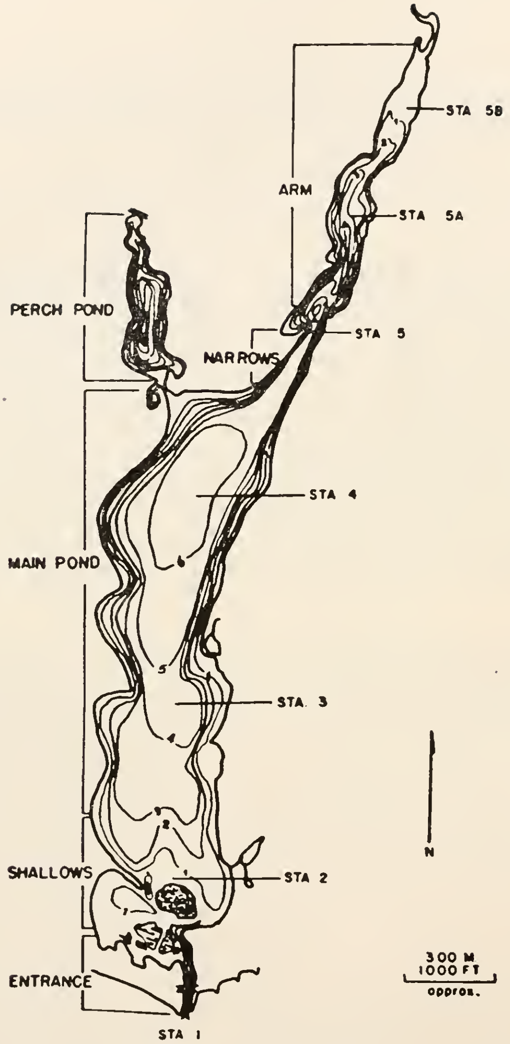

STA 5B
ARM
STA 5A
PERCH POND
STA 5
NARROWS
STA 4
MAIN POND
6
5
STA. 3
4
3
2
SHALLOWS
1
STA 2
N
ENTRANCE
300 M
1000 FT
approx.
STA 1

FIGURE 1. The morphometry of Great Pond. Depths are in feet.

diameter of 0.5 μ, manufactured by the Selas Corporation. Occasionally counts were made without filtration. Comparison of filtered and unfiltered counts showed that a large proportion of the cells was lost in filtration. Therefore, all filtered counts were multiplied by 1.81 to give the true concentration of cells in the water. All cells from 2–3 μ in diameter to about 50 μ in diameter were counted.

RESULTS

Figure 2 shows the distribution of total phytoplankton. In the surface waters greater concentrations occurred within the Pond, and this difference was greater

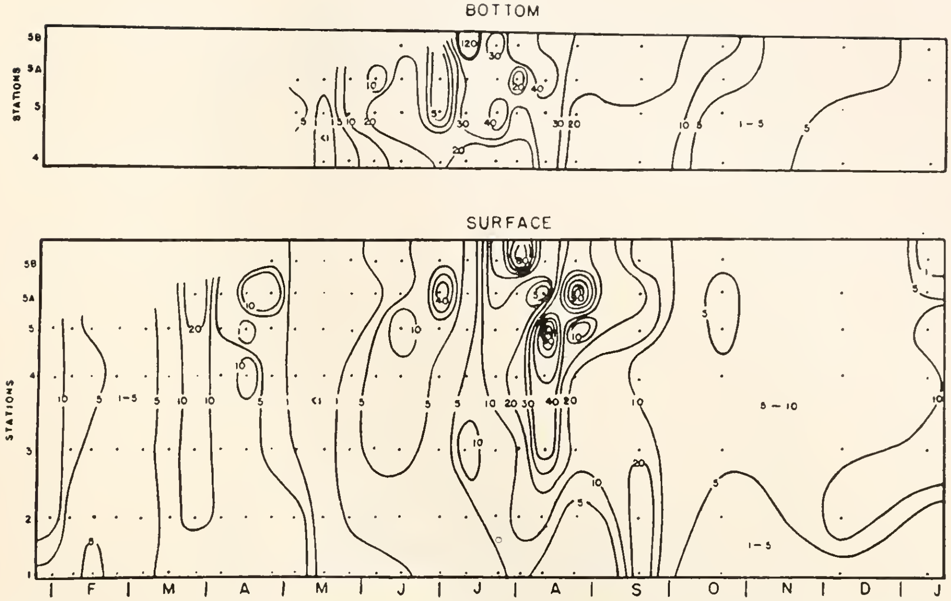


FIGURE 2. The distribution of total phytoplankton. Values × 10^6 are numbers of cells per liter.

during the summer than during the rest of the year. In summer there was, at a maximum, a 20-fold increase from the Sound to the surface waters of the arm. Between mid-September and the end of May, by contrast, the greatest increase from Sound to Pond was four-fold. The highest concentration in the surface waters, over 90,000,000 cells per liter, occurred in the summer at Station 5, well up the Pond. It is noteworthy that much greater fluctuations in cell concentrations occurred during summer than during the rest of the year. This feature was particu-

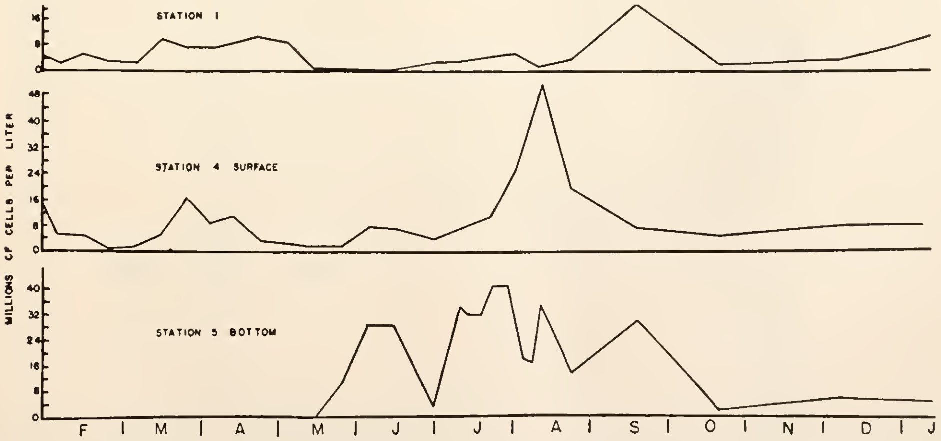
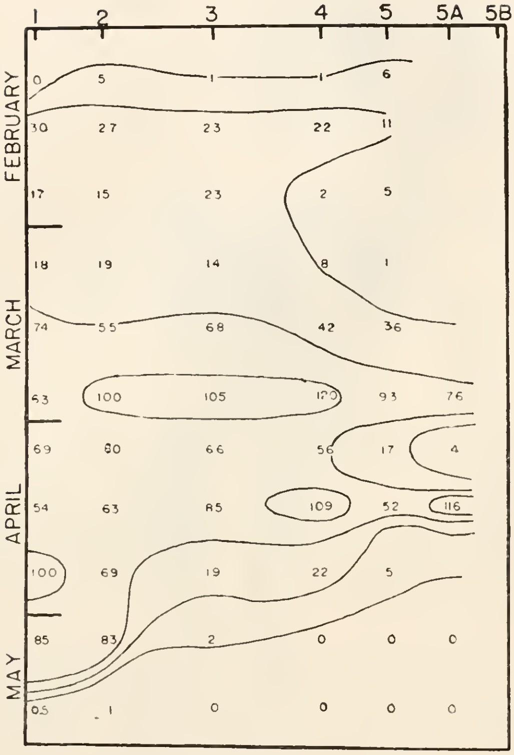
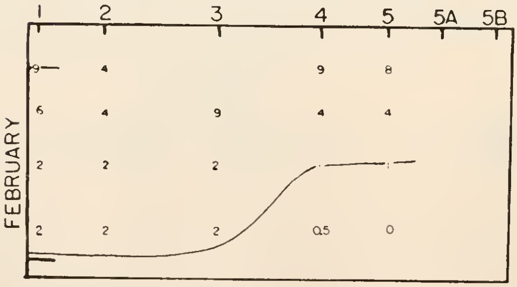


FIGURE 3. The seasonal cycle of total phytoplankton.

LEPTOCYLINDRUS MINIMUS



NITZSCHIA DELICATISSIMA



CHAETOCEROS DEBILIS

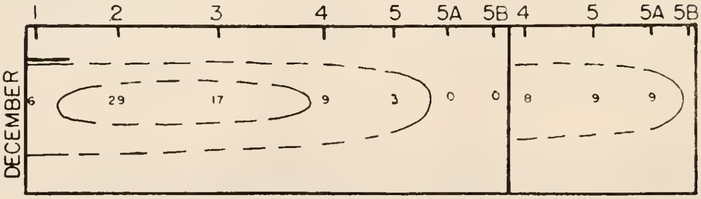


FIGURE 4. The distribution of Sound-derived species. Values × 10^5 are numbers of cells per liter. Station numbers, 1–5b, cross the figure.

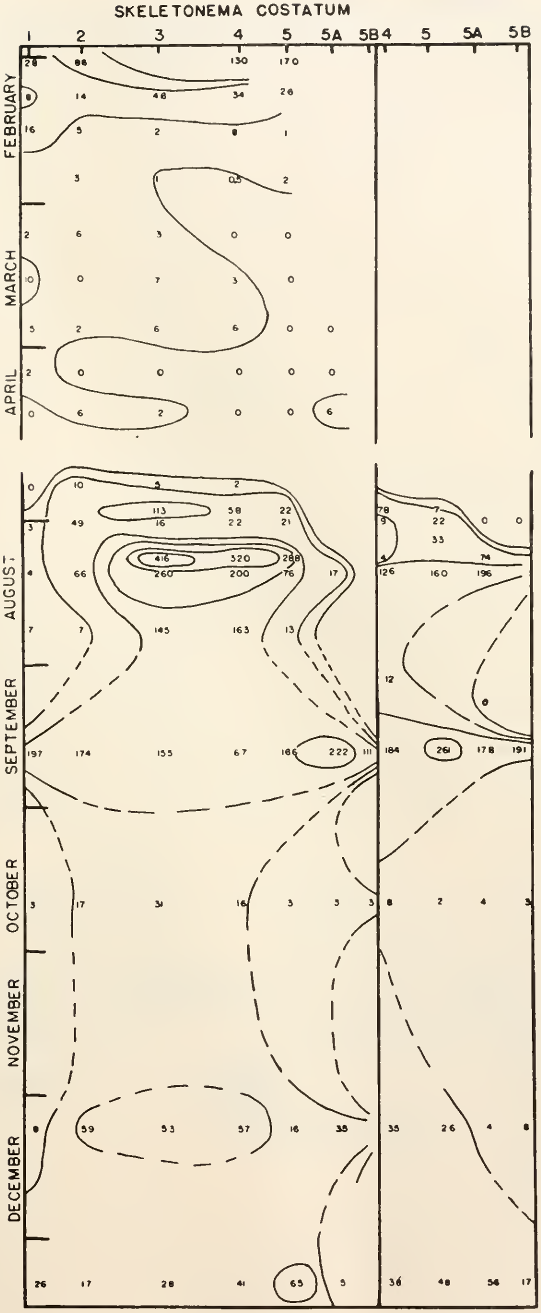


FIGURE 5. The distribution of *Skeletonema costatum*. Values × 10^5 are numbers of cells per liter. Station numbers, 1–5b, cross the figure.

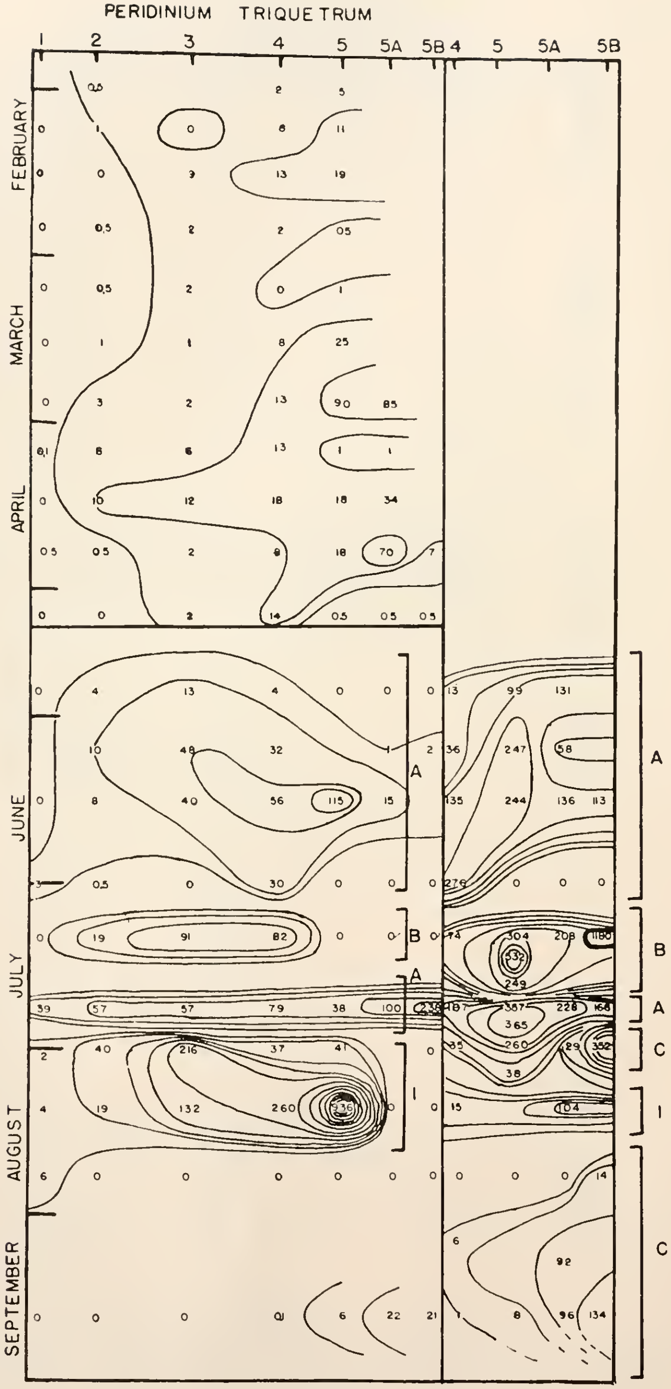

PERIDINIUM TRIQUETRUM
1 2 3 4 5 5A 5B 4 5 5A 5B
FEBRUARY
MARCH
APRIL
JUNE
JULY
AUGUST
SEPTEMBER
0.5 2 5
0 1 0 8 11
0 0 3 13 19
0 0.5 2 2 0.5
0 0.5 2 0 1
0 1 1 8 25
0 3 2 13 90 85
0.1 6 6 13 1 1
0 10 12 18 18 34
0.5 0.5 2 8 18 70 7
0 0 2 14 0.5 0.5 0.5
0 4 13 4 0 0 0 13 99 131
10 48 32 1 2 36 247 58
0 8 40 56 115 15 135 244 136 113
3 0.5 0 30 0 0 0 276 0 0 0
0 19 91 82 0 0 0 74 304 208 1180
532
249
39 57 57 79 38 100 236 187 387 228 168
365
2 40 216 37 41 0 35 260 129 352
38
4 19 132 260 936 0 0 15 104
6 0 0 0 0 0 0 0 0 0 14
6
92
0 0 0 0.1 6 22 21 1 8 96 134
A
B
A
I
A
B
A
C
I
C

FIGURE 6.

larly noticeable in the surface waters of the arm, where isolated pools of high concentrations occurred on June 30 and on August 1, 11, and 23.

Cell concentrations along the bottom were, in a gross way, similar to those at the surface, being greater in summer than in fall or winter. However, with the exception of a brief period at the beginning of July, cell concentrations remained at high levels throughout the summer. The highest concentration observed, 120, 000,000 cells per liter, was at Station 5b, far from the Sound.

Seasonal cycles for several stations are shown in Figure 3. In the Sound there was a bimodal cycle, with moderate concentrations in late winter and spring and high concentrations in early fall. The surface waters of mid-pond showed a bimodal curve, also, with roughly the same concentrations in late winter and spring, as in the Sound, but, unlike the Sound, with very high concentrations in later summer. Deep water at mid-pond had high concentrations throughout summer and September and moderate concentrations in fall and winter. Sound and Pond both showed lowest concentrations at the end of May.

Figure 4 shows the distributions of three species of diatoms, *Leptocylindrus minimus, Nitzschia delicatissima,* and *Chaetoceros debilis.* These were observed only during the cooler months and were found to extend from the Sound far into the Pond. *Leptocylindrus minimus* showed a relatively uniform distribution in the Sound and seaward portion of the Pond. Though concentrations were occasionally somewhat greater in the Pond than in the Sound, they never showed a variation that was more than two-fold. In the surface waters of the arm concentrations were about the same as those in the seaward portion of the Pond on days of upwelling, March 26 and April 15, but were much lower on a normal day, April 5, when no upwelling occurred. In the Sound and main pond *Leptocylindrus minimus* increased rapidly to become the dominant species by February 15, attained a maximum on March 25, and decreased abruptly early in May. *Nitzschia delicatissima* showed a similar distribution to *Leptocylindrus minimus* during February. Its concentrations were about one-tenth those of *Leptocylindrus minimus. Chaetoceros debilis* resembled *Leptocylindrus* in distribution.

Skeletonema costatum, the distribution of which is shown in Figure 5, was the only species of diatom that was present during summer as well as during the rest of the year. At times it exhibited a uniform distribution from the Sound far into the Pond, as on September 18, January 10 and February 25. At various other times it was more abundant in the Pond than in the Sound, the variation being eight-fold on October 20, December 7 and January 30 and as high as one hundred-fold in August. During fall and winter it was the dominant species, attaining a maximum on September 18. In February it declined as *Leptocylindrus minimus* became the dominant species. Although *Skeletonema* was not clearly a dominant organism in August, it reached its greatest concentration at that time.

In spring the dinophycean *Peridinium triquetrum* rose to considerable prominence (see Figure 6), its concentrations being as high as one-fifth of those of *Leptocylindrus* on April 15. It was more abundant within the Pond than in the Sound. There was a hundred-fold increase from the Sound to the mid-reaches of

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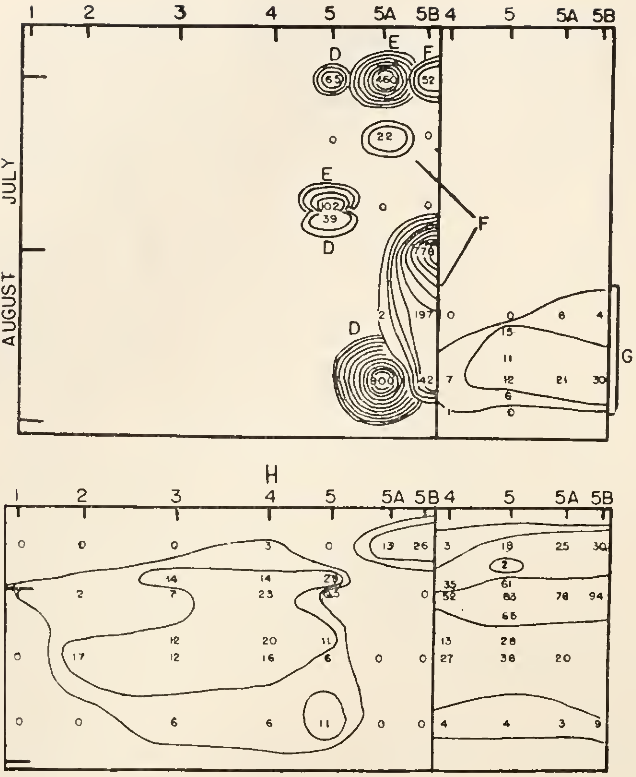
FIGURE 6. The distribution of Pond-derived species. Values × 10^5 are numbers of cells per liter. Station numbers, 1–5b, cross the figure. A represents undetermined chrysophycean, B *Bipedinomonas pyriformis,* C *Nephrochloris salina,* and I *Chaetoceros simplex.*



THALASSIOSIRA NANA

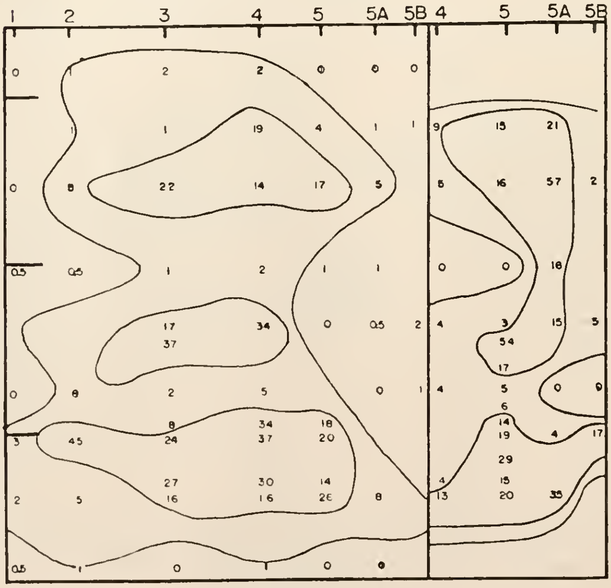


Figure 7.

the Pond on March 26. This difference was greater than any observed for the four diatoms so far described in the period between mid-September and the end of May.

Figure 6 shows the distributions of several species found primarily in summer. They were always far more abundant in the Pond than in the Sound. During June the dominant species was an undetermined chrysophycean flagellate. Its numbers were usually greater along the bottom than at the surface. The surface waters at the ends of the Pond had smaller concentrations than the waters of mid-pond, the difference being as much as one hundred-fold. Only in mid-pond did concentrations approach those along the bottom. Abrupt decrease of the chrysophycean was followed in early July by a growth of the flagellate *Bipedinomonas pyriformis.* Its distribution was identical to that of the chrysophycean. By late July *Bipedinomonas* had disappeared completely and the undetermined chrysophycean flagellate was dominant again throughout the Pond. On July 22 the chrysophycean showed an exceptional abundance at the surface of the arm due to upwelling. In August the small diatom *Chaetoceros simplex* became extremely abundant, more so in the surface waters of mid-pond than in the deeper water. On August 11 it showed a two hundred-fold difference between Sound and Station 5. The flagellate *Nephrochloris salina* was moderately abundant in August and September in bottom water at the extreme landward end of the Pond.

Figure 7 shows the distributions of several less important species of summer. The flagellate *Gyrodinium estuariale* and the minute diatom *Thalassiosira nana* exhibited the same type of distribution as described for the undetermined chrysophycean. In the surface waters of the arm contours are close-packed around the flagellate *Chlamydomonas* in the surface waters at the outlet of the river and around the flagellates, *Massartia rotundata* and *Rhodomonas minuta,* farther down the arm. The growth of these species was sporadic and attained very great concentrations. It is noteworthy that when these species grew in the surface waters, no traces of the species were found in the bottom water only five or six feet below.

Discussion

The species can be divided into Sound-, Pond-, and River-derived types. The Sound-derived type was abundant in the Sound and Pond alike. The Pond-derived type was abundant only in the Pond, particularly in the inner reaches and along the bottom. The river type was found primarily in the surface water at the outlet of the River. The two principal Sound-type species were the diatoms *Leptocylindrus minimus* and *Skeletonema costatum. Nitzschia delicatissima* and *Chaetoceros debilis* represented well-defined Sound types, but were of minor importance. Principal Pond types were the undetermined chrysophycean flagellate; the heterokontan flagellate *Nephrochloris salina;* the chlorophycean flagellate *Bipedinomonas pyriformis;* the dinophycean flagellates *Peridinium triquetrum, Massartia rotundata,* and *Gyrodinium estuariale;* the cryptophycean flagellate *Rhodomonas minuta;* and the diatoms *Chaetoceros simplex* and *Thalassiosira nana.* The River type was repre-

FIGURE 7. The distribution of Pond- and River-derived species. Values $\times 10^5$ are numbers of cells per liter. Station numbers, 1–5b, cross the figure. D represents *Massartia rotunda,* E *Rhodomonas minuta,* F *Chlamydomonas* sp., G *Gyrodinium dominans,* and H *Gyrodinium estuariale.*

sented by chlorophycean flagellates, consisting of two unidentified species of *Chlamydomonas*.

The Sound-derived species thrived during fall, winter, and spring primarily and in summer to a small degree. The Pond-derived species flourished in summer primarily and in spring to a small degree. All of the Sound-derived forms were nonmotile; the majority of the Pond-derived forms were motile.

During the fall, winter, and spring the water of the Sound, which fills 83% of the Pond at any time, carried its plankton far into the Pond. Within the estuary changes in this plankton were slight or negligible, indicated by the small difference in total plankton between Sound and Pond, by the change from dominance of *Skeletonema* to *Leptocylindrus* simultaneous in Sound and Pond, and finally by the intrusion of Sound species far into the Pond. During summer the Sound water carried with it little plankton, and once within the estuary the identity of this plankton was lost to a large degree. Plankton from the River could be identified only in the surface waters where the River emptied into the Pond. Throughout the greater portion of the Pond an indigenous plankton flourished.

TABLE I

Concentrations of diatoms and flagellates and statistics of physical features along the length of Great Pond

	Diatoms × 10^4/l	Flagellates × 10^4/l	Six-hour mean tidal velocity, knots	Salinity difference between surface and bottom, ‰
Sound	34	43	1.60	—
Mid-Pond, Station 4	633	598	0.01	6.38
Arm, Station 5a	319	1492	0.02	17.90

During the transport of water through the Pond, rapid changes in the plankton occurred in summer, as indicated by the abrupt changes in dominant species from place to place on the same survey. In the surface waters of the arm such changes caused large fluctuations in total phytoplankton. These changes indicate a rapid growth rate. During the remainder of the year abrupt changes in dominant species and extreme variations in total phytoplankton did not occur and hence growth rates were less than in summer. Thus, when conditions for growth in the Pond were good, Pond species rather than Sound species flourished. When growth conditions were less favorable, during the cool months, the plankton of the Sound drifted into the Pond, replacing the endemic flora that had previously flourished.

It has been pointed out that during summer the dominant members of the plankton included seven flagellates and three diatoms. In order to account for the preponderance of motile forms within the Pond, its association with low current velocities and great vertical stability of the water will be investigated. Averages of the dominant species as flagellates or diatoms are given for various stations in Table I. Surface and bottom values are averaged for stations 4 and 5a. Current velocities were taken from surface current measurements for Vineyard Sound one mile south of Great Pond (U. S. Coast and Geodetic Survey, 1949) and were computed for Great Pond by dividing the intertidal volume landward of a station by the cross-sectional area at the station. Surface current measurements may be higher than computed currents estimates by as much as 50% (Riley, 1952). The salinity difference between surface and bottom was the average difference of all observations during summer. Both diatoms and flagellates increased from the Sound to mid-pond, but flagellates increased at the expense of the diatoms from mid-pond to the headwaters. Tidal currents were much less from mid-pond to headwaters than in the Sound; while the vertical salinity gradient became progressively greater up-pond.

The moderate success of the motile cell from Sound to mid-pond can be attributed to a decrease in vertical turbulence, as a result of lessened tidal current. The greater success from mid-pond to the headwaters can be attributed to further decrease in turbulence, as a result of both weak currents and marked stability. Settling out of non-motile cells in the quiet waters of the Pond, particularly the arm, would place the motile cell at an advantage. In addition, cells destined to settle out would have less time to divide and replace the stock in the shallow waters of the Pond than in the deep waters of the Sound, so that the motile cell would be at a still greater advantage in the Pond. Thus, when growth conditions favor an endemic flora, the quietness and shoalness of the Pond water should favor motility as a characteristic of the flora.

The Pond flora may have been better adapted than the Sound flora to lowered salinity, high temperature, or nutrient products of the intermediate metabolism of the pond water. Adaptations of these sorts may have been effective in favoring the successive blooms of different species and in fostering the occasional dominance of non-motile forms.

In conclusion, the summer conditions at Great Pond furnish an example of the dominance of flagellates rather than diatoms in the phytoplankton. This dominance appears to result in part from a coupling of good growth conditions, which foster an endemic flora, with a quiet, very shallow habitat, which should favor the non-settling, motile cell.

The author wishes to express the greatest gratitude to Dr. A. C. Redfield and to Dr. W. R. Taylor for their help during this study. The best of thanks go to Dr. John Barlow, for use of salinity data and help in collecting samples, and to Mr. Harry Turner, for use of the chart of Great Pond prepared by him.

Summary

1. Determinations of phytoplankton concentrations were made throughout 1950 in Great Pond, a small estuary near Falmouth, Massachusetts.

2. Greater concentrations occurred within Great Pond than in the ocean water at its entrance. This difference was greatest in summer.

3. During fall, winter, and spring the same species populated the waters of the ocean and the Pond, but during the summer an assemblage of species flourished within the Pond that was never found in the ocean water and was not characteristic of the river water.

4. The Pond-derived flora of summer contained a large proportion of flagellates, whereas the Sound-derived flora was composed completely of diatoms.

5. The preponderance of motile forms within the Pond during summer was attributed to good growth conditions, which stimulated an endemic flora, and to shoal, quiet water, which should favor the non-settling type of cell.

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ON THE CHEMICAL COMPOSITION OF THE BROWN ALGA HIMANTHALIA ELONGATA (L.) S. F. GRAY

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In recent years the commercial importance of many seaweeds has resulted in considerable work being carried out on the chemical composition of marine algae (Black, 1953). *Himanthalia elongata,* although a common dioecious fucoid along the coasts of northern England, does not occur in sufficient quantities to render it of commercial importance. As a result it has been little investigated. Colin and Ricard (1930) first recorded the chemical composition of *Himanthalia,* but their work is of little value since no indication of the size or condition of the plants analyzed was given. Moss (1952), however, noted that during the spring a variation in chemical composition occurred with the stage of development of *Himanthalia* collected at the same time from the same habitat. No distinction of sex was made in the samples, although for *Fucus vesiculosus* Moss (1950) found that the male receptacles were higher in total nitrogen than the female receptacles.

The present work is concerned with the chemical constituents of both male and female plants of *Himanthalia* and with the variation of such constituents along the length of the plant.

Materials and Methods

Samples of *H. elongata* were collected from St. Mary's Island, Northumberland at low water as the tide receded. All samples were returned to the laboratory in sea water thus ensuring that the plants, prior to analysis, were fully turgid. For analysis samples were sorted into developmental and sexual stages. Each sample contained at least 100 individuals. Fresh weight determinations were made after removing the adhering moisture from the surface of the plants with cheese cloth. The plant material was loosely packed in shallow aluminum trays, placed in an electric oven through which a current of air was forced. The material was dried for one hour at 90° C. (to inactivate enzymes) and then for a further 23 hours at a temperature range of between 60 and 65° C. On cooling to room temperature the dried material was ground in a Christie & Norris No. 8 Laboratory Mill, fitted with a $\frac{1}{64}$-inch perforated screen, giving a powder which practically all passed 90 mesh. Dry weight determinations were made on 0.1–0.2 gm. samples of the above dried milled weed.

Total nitrogen estimations were obtained by the micro-Kjeldahl method of Kelley, Hunter and Sterges (1946) using a Markham Still for distillation. The soluble non-protein nitrogen was extracted from the dried material with water for 6 hours at laboratory temperature with constant shaking, then stored at 5° C. for 18 hours with occasional shaking. After filtering, the proteins were precipitated with sufficient trichloracetic acid to produce a 2.5% solution. The extract was filtered through Whatman 42 filter paper and made up to 250 ml. This method of extraction gave consistent results for total non-protein nitrogen. The soluble non-protein nitrogen was determined using the method of Langer (1952). Of the soluble non-protein constituents, volatile base and amide nitrogen values were obtained by the method of Channing and Young (1953). The free α-amino nitrogen was estimated using the technique employed by Sobel, Hirschman and Besman (1945). Peptide nitrogen was determined using the method of Haas, Hill and Russell-Wells (1938); Sideris and Young (1946).

The carbohydrate and related constituents were determined by the methods of Cameron, Ross and Percival (1948).

Inorganic ash content of the dried material was estimated after preliminary sulphuric acid treatment as described by Piper (1950).

Free amino acids were extracted from the dried milled weed (one gm.) by shaking three times with 75% ethanol (100 ml.). The solution was centrifuged after each extraction and the combined extracts passed through Zeo-Karb 225 and the free amino acids and peptides eluted with ammonium hydroxide (1 N). The eluates were dried *in vacuo* over calcium chloride and taken up in HCl (0.1 N) and chromatographed. Combined amino acids in the dried material (0.5 gm.) were hydrolyzed in a sealed tube with HCl (6 N; 5 ml.) for 48 hours at 105° C. Excess acid was removed by drying *in vacuo* over calcium chloride and sodium hydroxide. Samples were ion exchanged as above and similarly chromatographed. For the detection of tryptophane, alkaline hydrolysis with barium hydroxide (saturated at room temperature) was used.

Free sugars were extracted from the dried material (3 gm.) after refluxing with 75% ethanol (100 ml.) for 6 hours. After filtration the extract was passed through the ion exchange resins Zeo-Karb 225 and De-Acidite E, reduced in volume and aliquots used for paper chromatography. Sugars on hydrolysis were determined by hydrolyzing the dried milled weed (one gm.) with HCl (50 ml.; 2 N) for 8 hours using a reflux condenser. Following filtration the extract was subjected to ion exchange resins as for free sugars and chromatographed.

Extraction of the organic acids was accomplished by shaking the dried milled weed (3 gm.) with water (100 ml.) for 6 hours. The mixture was filtered and 80% ethanol added to precipitate protein. The precipitate was removed by centrifugation and the supernatant passed through Zeo-Karb 225 followed by De-Acidite E. The acidic compounds were eluted from the latter resin with ammonium hydroxide (1 N).

Methods in paper chromatography were those described by Block (1952) and Ranson (1955).

RESULTS

1. *Qualitative determination of some chemical constituents*

a) Amino acids, free and combined

The presence of only a few free amino acids, namely alanine, aspartic and glutamic acids, was detected on one and two dimensional chromatograms. The normal plant amides asparagine and glutamine were not detected. On hydrolysis with HCl (6 N) for 48 hours the alcoholic extracts gave compact spots corresponding to aspartic, glutamic, alanine and traces of glycine, serine and the leucines, suggesting the presence of these acids in peptide formation. The intensity of the glutamic and alanine spots was greatly increased after hydrolysis, suggesting that they occur in peptides as well as in the free state. Other alcoholic extractions of different developmental stages of *Himanthalia* gave similar results.

A chromatographic analysis of the peptide fraction precipitated from the water-soluble non-protein extracts with mercuric acetate and treatment with H_2S, as employed by Haas, Hill and Russell-Wells (1938), after hydrolysis revealed the presence of the following amino acids: glutamic (strong), alanine (strong), aspartic (weak-strong), serine (weak), glycine (weak), leucine (trace), isoleucine (trace).

In acid and alkaline hydrolysates of the dried milled weed from all stages of development the following amino acids were detected: alanine, arginine, aspartic, cysteic, glycine, glutamic, histidine, iso-leucine, leucine, lysine, methionine, methionine sulphoxide, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine. In the majority of samples analyzed histidine was present only in traces, its presence being confirmed with the Pauly diazotinized sulphanilic reagent (Consden *et al.*, 1946). The isatin dip method of Jepson and Smith (1953) confirmed the presence of proline and the absence of hydroxyproline. The copper carbonate technique for the detection of non α-amino acids (Crumpler and Dent, 1949) failed to show the presence of either β-alanine or γ-amino butyric acid in the free state or in combination.

b) Carbohydrates

Very little sugar could be detected in the free state. Spots with R_F values similar to fucose and galactose were obtained. Glucose, fucose, arabinose, xylose, and galactose were found to be present on chromatograms of the acid-hydrolyzed weed samples. The sugar alcohol mannitol was detected on both sugar chromatograms.

c) Organic acids

Very few organic acids were detected in extracts of *Himanthalia*. Citric acid was the chief organic acid detected. In some cases traces of malic acid were found to be present. No other acids of the Krebs cycle were detected. On all chromatograms a large acidic spot was found to exist in appreciable quantity. In tertiary amyl-formic acid-water solvent, the $R_F = 0.20$, in the propanol-NH_3 solvent, the $R_F = 0.51$. With silver nitrate reagent it gave an intense white color on a brown background. It was negative to ninhydrin and Hane's phosphate reagent. On the chromatograms it ran in close association with standard gluconic acid. The large quantities of alginic acid present in the weed samples leads one to think that it may be mannuronic acid, which would be the precursor of the larger alginic acid molecule. Unfortunately a standard sample of mannuronic acid could not be obtained. Another acidic spot was found to be orthophosphate.

2. *Variation in nitrogenous constituents*

During July, 1953, samples of *Himanthalia* were collected from St. Mary's Island. After preliminary sorting, drying and grinding these plants were analyzed

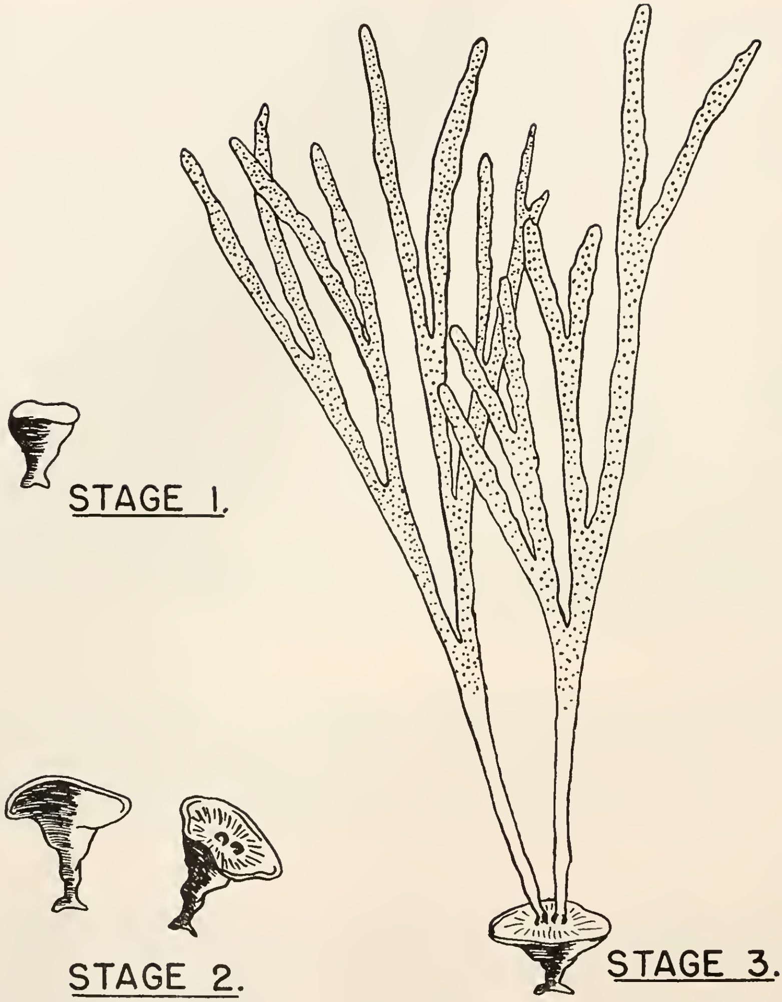

STAGE 1.
STAGE 2.
STAGE 3.

Figure 1. Stages of development of Himanthalia used in the chemical analysis, July, 1953: Stage 1. Young vegetative buttons, 1–1.5 cm. in length and tubular in form. Stage 2. Fully grown vegetative buttons. Stage 3. Mature plants composed of buttons, from which were given off long mature receptacles some 50–60 cm. in length. The receptacles were separated from the buttons, and further sorted into male and female for separate analysis.

for total nitrogen, protein nitrogen and non-protein nitrogen. The stages of development used in this analysis are shown in Figure 1.

The results (Table I) are interesting insofar as they show that the male receptacles contain higher nitrogen values than the female receptacles. The higher nitrogen content of the male is associated with the protein fraction. The vegetative plants, *i.e.,* the buttons, contain a higher percentage of soluble non-protein nitrogen than the mature buttons in stage 3. This appears to consist mainly of peptide and free amino acid nitrogen.

TABLE I

Variation of nitrogen in plants from St. Mary's Island, July, 1953

i) Total nitrogen, protein and non-protein nitrogen

Stage of development	Total N	Protein N	Non-protein N	Non-protein N as % total N
1. Buttons	6.75	5.84	0.91	13.5
2. Buttons	12.30	9.85	2.45	19.9
3. Buttons	12.15	10.92	1.23	10.1
Female receptacles	13.45	10.54	2.91	21.6
Male receptacles	15.00	12.12	2.89	19.3

ii) Soluble non-protein constituents

Stage of development	Volatile base N	Peptide N	α-Amino N
1. Buttons	0.20	0.46	0.43
2. Buttons	0.62	1.39	0.59
3. Buttons	0.31	0.97	0.32
Female receptacles	0.63	1.54	0.93
Male receptacles	0.60	1.40	0.69

3. *Further analysis of male and female receptacles*

During September, 1954, when plants were ripe, male and female individuals were collected and the receptacles analyzed separately. The results are given in Table II.

In order to obtain antherozoids and oogonia for analysis, male and female receptacles were separately washed with filtered sea water and allowed to dry out for several hours. During this process gelatinous masses of gametes appeared at the ostioles of the conceptacles. The plants were then placed in separate Pyrex glass containers with filtered sea water and the plants allowed to extrude their gametes. The gametes were separated from the sea water by centrifugation, dried at 90° C.

TABLE II

Analysis of male and female receptacles, Sept., 1954

	Male receptacles		Female receptacles	
Mean height	84.7	cm. per unit plt.	86.5	cm. per unit plt.
Mean width	0.8	cm. per unit plt.	0.8	cm. per unit plt.
Fresh weight	122.3	gm. per unit plt.	119.0	gm. per unit plt.
Dry weight	15.6	gm. per unit plt.	15.2	gm. per unit plt.
Total nitrogen	19.7	mg. per gm. dry weight	16.6	mg. per gm. dry weight
Protein nitrogen	14.8		12.0	
Non-protein N	4.9		4.6	
Mannitol	65.1		66.8	
Alginic acid	132.4		178.9	

TABLE III

Analysis of male and female gametes, Sept., 1954

Constituent	Male gametes (mg./gm. dry wt.)	Female gametes (mg./gm. dry wt.)
Total nitrogen	69.33	25.97
Protein nitrogen	55.81	13.83
Non-protein nitrogen	15.52	12.14
Total ash	826.60	819.60

for one hour, followed by 23 hours at 60° C. An analysis of the nitrogenous constituents and total inorganic ash is given in Table III.

The results of these two analyses show that between the male and female receptacles there is little difference in size and weight. The protein nitrogen is higher in the male than in the female, thus confirming the results found for the receptacles collected in July, 1953. The male gametes are appreciably higher in protein content than the female gametes, which suggests that the high protein content of the male receptacles is in all probability due to the high protein content of the gametes. The exceptionally high ash content of the gametes is of great interest. The alginic acid is higher in the female receptacles than in the male.

4. *Variation of chemical constituents along the length of the plant*

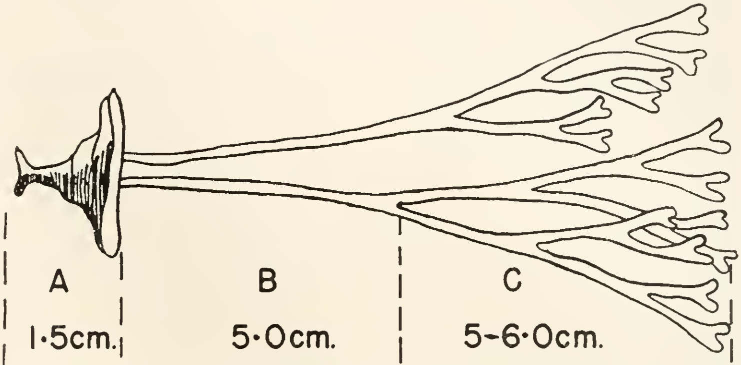
One hundred plants were collected from St. Mary's Island during April, 1954, and divided into three regions for analysis of the various chemical constituents. The divisions were as follows:

Region A. Mature buttons of the plant.
Region B. Sterile base of receptacle, the length of which was approximately 5 cm. from the surface of the button.
Region C. Remainder of the receptacle. This region contained developing conceptacles which are differentiated behind the growing apices. Length of region varied from 5.0 to 6.0 cm.

The results in Figure 2 indicate that variations occur along the length of the plant. Of the nitrogenous constituents analyzed, the young developing receptacle tips and conceptacles of region C possess higher values than the base of the receptacle (region B) which is sterile. The vegetative button (region A) possesses considerably lower amounts of nitrogen than those recorded for either region of the receptacle. The tips and base of the receptacle are higher in mannitol than the vegetative button. The button, however, is higher in alginic acid and laminarin content. The sterile base of the receptacle contains more alginic acid than the tips of the receptacles.

GENERAL DISCUSSION

The qualitative determination of some of the chemical constituents recorded for *Himanthalia* is in accordance with the findings of other workers. The detection of only a few amino acids in the free state is to be found in other marine algae (Channing and Young, 1953; Coulson, 1953a; Smith and Gordon Young, 1953). The presence of peptides, particularly of glutamic acid and alanine, affords further evidence that marine algae differ from fresh water algae and land plants in that they show a preference for peptides in place of amino acids (Dent, Stepka and Steward, 1947; Steward and Thompson, 1950; Fowden, 1951). The typical land plant



CONSTITUENT	BUTTON	BASE of RECEPTACLE	UPPER RECEPTACLE
TOTAL N	13·51	20·25	28·70
PROTEIN N	11·87	15·56	20·29
NON PROTEIN N	1·64	4·79	8·41
VOL. BASE N	0·39	0·66	0·77
FREE AMINO N	0·50	1·20	1·19
PEPTIDE N	0·71	1·59	2·12
ALGINIC ACID	215·40	166·80	119·00
MANNITOL	60·77	74·38	79·62
LAMINARIN	21·18	8·15	9·61

(mg. per gm. dry wt.)

FIGURE 2. Variation of constituents along length of plant, April, 1954.

amides asparagine and glutamine were not detected in *Himanthalia.* The amino acids present in the hydrolysates of the marine alga were similar to those found by other workers for the Phaeophyceae (Channing and Young, 1953; Coulson, 1953b; Smith and Gordon Young, 1953), and for fresh water algae (Fowden, 1954). They are similar to those in land plants. The sugars fucose and galactose, and the sugar-alcohol mannitol were detected in the free state. The recent work of Lindberg (1953) and Lindberg and Paju (1954) has shown the presence of mannitol and mannitol glucosides to occur in other fucoids. It is realized, however, that the presence of fucose and galactose may be the result of polysaccharide hydrolysis during the drying process. The sugars glucose, fucose, arabinose, xylose and galactose present in the acid hydrolysates are in keeping with the results of other workers (Connell, Hirst and Percival, 1950; Percival and Ross, 1950; Ross, 1953; Dewar, 1954), in that they are the component sugars of laminarin, fucoidin and cellulose. Little work has been published on the organic acids of marine algae apart from the work of Créach (1952), who detected citric acid in a number of fucoids. The presence of citric acid and traces of malic acid found in *Himanthalia* are commonly found in land plants. The presence of free orthophosphate has been noted for other algae (Blinks, 1951).

The male receptacles of *F. vesiculosus* were shown by Moss (1950) to be higher in nitrogen content than the female receptacles. For *Himanthalia* similar results have been obtained, the male receptacles containing more nitrogen, particularly protein nitrogen, than did the female receptacles. The male gametes of *Himanthalia* were also found to contain more nitrogen than female gametes. This is similar to results published by Sosa-Bourdouil (1940), who, for *F. vesiculosus,* found the male gametes to contain twice as much nitrogen as the female gametes on a dry weight basis. The high concentration of nitrogen in the male gametes seems therefore to be the factor contributing to the high nitrogen content of the male receptacles.

The analysis of the chemical constituents along the length of the plant showed the tissues of mature buttons to be lower in nitrogen and mannitol but higher in alginic acid and laminarin than were the receptacles on the same plant. The apical regions of the receptacles were high in mannitol and nitrogen content, particularly protein. The basal sterile region of the receptacle was similarly high in mannitol, but lower in nitrogen than the uppermost region. The higher soluble non-protein nitrogen content of the upper receptacle region is, no doubt, correlated with the synthesis of protein for growth and gamete formation. The results may be compared with those of Moss (1950), who for *F. vesiculosus* found that the young receptacles were higher in nitrogen content than the vegetative regions of the thallus, which is in contrast with *Himanthalia* for in this species the fertile tissue was higher in nitrogen content than the vegetative button. Jacobi (1954) has recorded high nitrogen values for the apical regions of *F. vesiculosus* throughout the year. For *Laminaria saccharina,* the same author found a higher nitrogen content in the meristematic region of the thallus than in the more mature tissues of the frond. The findings of Moss (1950) and Jacobi (1954), together with the present author's results for *Himanthalia,* indicate that actively growing regions and regions of reproductive development are higher in nitrogen content than the more mature vegetative tissues of the plant.

The work in this paper forms part of the programme of research and development on marine algae by the Institute of Seaweed Research and the author is indebted to the Institute for permission to publish. The author also wishes to thank Dr. Betty L. Moss, under whose supervision this work was carried out.

SUMMARY

1. The qualitative determination of amino acids, sugars and organic acids in *Himanthalia elongata* has been carried out.
2. Mature vegetative tissues were found to be lower in nitrogen content than actively growing reproductive tissues.
3. The high nitrogen content of the male receptacles was correlated with the higher protein nitrogen content of the male gametes.
4. Vertical gradients of organic constituents were found to occur along the length of the individual plant. The mature button was lower in nitrogen and mannitol but higher in alginic acid and laminarin than the receptacles of the same plant. The apical regions of the receptacles were high in mannitol and nitrogen content, particularly protein. The basal sterile region of the receptacle was similarly high in mannitol, but was lower in nitrogen than the uppermost region.

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THE EFFECT OF SEX AND ALLATECTOMY ON THE OXYGEN CONSUMPTION OF THE THORACIC MUSCULATURE OF THE INSECT, LEUCOPHAEA MADERAE[1]

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Previous results of metabolic studies in a variety of insect species clearly illustrate the difficulty in making generalizations (Sacktor, Thomas, Moser and Block, 1953). Thus it is not too surprising that, in contrast to the work of Barron and Tahmisian (1948) in *Periplaneta americana*, no sex difference was found in the present study regarding the endogenous oxygen consumption of the musculature of *Leucophaea maderae*. A further, more interesting result is that the thoracic muscles of both sexes of *Leucophaea maderae* increase their oxygen consumption after allatectomy. This finding is in seeming contrast to that of Thomsen (1949) who observed a decrease in the oxygen consumption of the whole intact fly, *Calliphora erythrocephala*, following allatectomy. However, this discrepancy may perhaps be explained on the basis of the work of L'Hélias (1953) who found an increase in the carbohydrate content of tissues and a decrease in blood sugar of *Dixippus morosus* after the removal of the corpora allata (see Discussion).

Preliminary tracer experiments were undertaken in order to ascertain whether there was any correlation, *in vivo*, between the increased oxygen consumption and phosphorylation, but they were terminated before any definite conclusions could be drawn.

METHODS

The determination of oxygen consumption (QO_2) was carried out at 26° C. in a conventional Warburg apparatus, each vessel containing a total volume (including muscle and KOH) of 3 ml. of Bělař's solution (.9% NaCl; .02% KCl; .02% $CaCl_2$) buffered at pH 6.8 with .01 *M* phosphate. The thoracic muscles, weighing approximately 150 mg., of a single animal were kept on ice (1–3° C.) in Bělař's solution, without glucose or other substrate, weighed, separated with the aid of needle probes into very small pieces, and placed in the Warburg vessels. Oxygen consumption measurements were initiated, after a 15-minute equilibration period, in less than an hour after the muscles were dissected out. The moisture content, determined on normal animals of both sexes and allatectomized animals at the end of a few of the Warburg runs, averaged 80%. Allatectomy was performed soon after emergence and the operated animals were used two to three months afterwards. The controls were always of the same adult age.

The preliminary tracer experiments were carried out *in vivo*. Two microcuries

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of a high activity solution of neutralized ortho sodium phosphate [32] were injected into the body cavity of each of the animals. They were sacrificed 24 hours later, the thoracic muscles and the gut were dissected out, rinsed in several changes of phosphate-free Bělař's solution, blotted, weighed, and frozen on dry ice, prior to homogenizing in iced 10% trichloracetic acid. The phosphorus was fractionated by the method described by Schneider (1945).

Counting was carried out with a thin window (1.4 mg.) G-M tube on aliquots of the ashed fractions, containing fairly uniform amounts of phosphate.

RESULTS

Table I shows that there was no significant difference between the sexes in the QO_2's of the thoracic muscles, 11 males averaging 3.25 ± .28 and 10 females averaging 3.35 ± .28 cubic mm./mgm. dry wt./hour. Deviation is expressed as standard error. Inasmuch as the oxygen uptake of the muscles was linear for about 50 minutes, the QO_2 values were calculated on the basis of the average oxygen consumption at 30 minutes. In a few trial experiments, we were able to confirm the results of Barron and Tahmisian (1948) on *Periplaneta americana,* in which they found a larger oxygen consumption in male thoracic muscles than in females.

TABLE I

Oxygen consumption of roach muscle Leucophaea maderae

Male versus Female

Experiment No.	Males QO_2	Females QO_2
1	2.7	2.9
2	2.5	2.1
3	3.0	2.5
4	2.8	3.9
5	1.7	4.6
6	4.7	3.3
7	3.9	2.3
8	4.1	4.1
9	4.0	3.1
10	4.2	4.7
11	2.2	
Average	3.25±.28	3.35±.28

In another experimental series dealing with allatectomized as compared with normal control males and females, the muscles of 18 normal animals had a QO_2 of 3.50 ± .22 while 18 allatectomized males and females averaged 4.26 ± .20. These results are shown in Table II. The 20% difference is significant, as a "t" test gives a P of approximately .02.

The two series of experiments (male vs. female and normal vs. allatectomized) were done at widely separate times, and as slight advantage, statistical or otherwise, would be gained by pooling the two series of normal animals, the calculation was not made.

In preliminary, *in vivo,* tracer experiments, utilizing P^{32}, the 24-hour uptake into the acid-soluble (A.S.), phospholipid (P.L.), nucleic acid (N.A.) and residual phosphate (R.P.) fractions was determined. Eight animals, five normal and three

TABLE II

Oxygen consumption of roach muscle Leucophaea maderae

Normal versus Allatectomized

Experiment No.	QO_2	
	Normal	Allatectomized
1	4.3	4.8
2	2.5	3.7
3	3.9	4.5
4	4.6	5.8
5	3.3	4.2
6	2.3	3.8
7	4.1	4.4
8	3.1	4.2
9	4.7	5.9
10	3.0	4.1
11	2.8	3.8
12	1.7	2.8
13	4.7	5.2
14	3.9	4.4
15	4.1	4.0
16	4.0	3.3
17	4.2	4.8
18	2.2	3.0
Average	3.50 ± .22	4.26 ± .20

allatectomized, were used. Tissues were rinsed thoroughly to remove adhering tracer phosphorus. In terms of relative specific activities, that is, specific activity ratios of the insoluble fractions to the acid-soluble fraction, the ratios in the gut were two to five times higher than those of similar fractions of muscle. The higher relative specific activities of gut indicate that phosphate is metabolized at a higher rate in all fractions of gut than in those of muscle. This higher metabolic activity is supported by preliminary experiments in which the oxygen consumption of gut was also shown to be higher than that of muscle. However, as it was difficult to be certain of complete removal of normal gut bacteria, the reliability of the QO_2 values was questionable at that time.

TABLE III

Uptake of radioactive phosphorus into muscle of normal and allatectomized Leucophaea maderae

Date	Animal	Specific activity							
		Acid soluble	A.S./Blood	P.L.	P.L./A.S.	N.A.	N.A./A.S.	P.P.	P.P./A.S.
1–14–54	Normal	255	.66	12.4	.048	41.0	.160	37.4	.146
1–14–54	Normal	246	.64	10.8	.044	44.6	.182	39.0	.158
1–14–54	Allatectomized	377	—	14.0	.037	61.0	.161	82.8	.218
7–9–54	Normal	105	.44	5.0	.047	16.6	.158	14.0	.133
7–9–54	Normal	293	.45	11.6	.040	48.0	.164	51.6	.178
7–9–54	Normal	134	.54	7.2	.053	24.2	.181	24.4	.182
7–9–54	Allatectomized	154	.55	6.9	.045	29.9	.194	28.0	.181
7–9–54	Allatectomized	165	.42	8.1	.049	41.0	.248	33.9	.206

However, in the muscles, as shown in Table III, no significant differences could be found in the AS or PL fractions between normal and allatectomized roaches. For example, the relative specific activities of the phospholipids PL/AS of the normals average .046 ± .004 while those of the allatectomized average .044 ± .005. Error is expressed here as standard deviation.

The specific activities of the N.A. and P.P. fractions appear closely related, which may mean that the two groups of compounds are metabolically related, that they have reached a similar equilibrium value, or that the method of separation is inadequate. However (although no statistical significance may be attached to the fact) the average of the relative specific activities of the allatectomized animals in the N.A. and the P.P. fractions, separately, is higher than that of the controls.

Discussion

The increased oxygen consumption of the muscles of the allatectomized animals (Table II) indicates that the removal of the corpus allatum hormone may result in an elevation of the endogenous substrate concentration (glucose and glycogen) and/or the concentration of some oxidative enzymes in insect muscle. In allatectomized *Dixippus morosus,* L'Hélias (1953) found an elevated carbohydrate content in the tissues and a lowered blood sugar. If comparison of these two forms of insect is possible, one would, by analogy, expect a similar increase in the carbohydrate stores in the muscles of allatectomized *Leucophaea maderae,* which when removed from the *in vivo* situation and thrown upon its endogenous resources *in vitro,* would result in a higher oxygen consumption than that of the normal control muscles under the same circumstances. Furthermore, if the lowered blood sugar of *Dixippus morosus* holds for *Calliphora* as well, it would explain the results of Thomsen (1949) showing a lowered oxygen consumption of living allatectomized flies, for in the living animal the tissues might be constrained to the use of the lowered blood sugar and would consequently respire at a lower rate than controls with a normal blood sugar.

This hypothesis is, of course, tentative in view of the metabolic variation obtaining even in closely related species of insects.

The absence of a sex-correlated difference in muscle oxygen consumption shown here, although not obtaining in *Periplaneta americana* (Barron and Tahmisian, 1948) could have been predicted from the succinoxidase contents of muscles of males and females of these species, *Leucophaea maderae* and *Periplaneta americana,* as the results of McShan, Kramer and Schlegel (1954) which appeared while this work was in progress showed that there is no sex difference in the content of this oxidative enzyme in *Leucophaea maderae,* as compared to the three-fold difference found by Harvey and Beck (1953) in *Periplaneta americana.*

Little may be said concerning the results obtained with tracer phosphorus, as the few experiments completed show no changes comparable to the oxygen consumption data. However, it should be recognized that these values represent only gross phosphate fractions. Neither specific activity-time relations nor the correspondence between *in vivo* and *in vitro* P^{32} uptake by this roach muscle was investigated.

Consequently the question of whether or not the increased oxygen consumption of muscle of allatectomized *Leucophaea maderae* is coupled to phosphorylation, must remain the subject of further research.

The author wishes to express his gratitude to Dr. Berta Scharrer for introducing him into the problems of insect physiology, for donating the animals, both normal and allatectomized, and for working with him in many of the experiments.

SUMMARY

1. Endogenous oxygen consumption of thoracic muscles of *Leucophaea maderae* was no higher in males than in females, but there was a significant increase in this parameter in allatectomized animals of both sexes.

2. Preliminary tracer phosphorus studies were made *in vivo* and the relative specific activities of the muscle fractions are shown.

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FERTILIZATION OF FERTILIZED SEA URCHIN EGGS[1]

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Experiments by Tyler and Schultz (1932) on eggs of the echiuroid worm *Urechis caupo* showed that repeated fertilization was possible. In these experiments development of the eggs was blocked by treatment with slightly acidified sea water within three minutes after the first insemination. When returned to normal sea water at 15 or more minutes later these eggs failed to resume development although containing a spermatozoon. In fact they assumed the appearance of unfertilized eggs. Upon re-insemination a second sperm entered and development proceeded with typical polyspermic cleavage and the formation of abnormal embryos. These experiments have been confirmed on eggs of *Urechis unicinctus* by Kagawa (1952).

In the above-described experiments the possibility of re-fertilization was apparently confined to the first few minutes after fertilization and before the time at which membrane elevation occurs in this species (see Tyler, 1932). More recently it has been shown by Sugiyama (1951) that sea urchin eggs which have been mechanically deprived of their fertilization membrane can be re-fertilized (becoming heavily polyspermic) at considerably later stages provided they are exposed for a short time to Ca- and Mg-free sea water or to isotonic urea. The re-fertilization could be accomplished as late as the two-cell stage. The treatment with Ca- and Mg-free sea water or with isotonic urea usually was for five minutes at a short time after the initial insemination. Sugiyama also reported that if the eggs are put in Ca- and Mg-free sea water before the fertilization membrane rises the latter elevates as a thin structure. When returned to normal sea water two to five minutes later and sperm added the membranes sink to the surface of the egg and re-fertilization occurs.

Hagström and Hagström (1954a) confirmed the re-fertilizability of fertilized sea urchin eggs, using trypsin-treatment of the unfertilized egg to inhibit membrane elevation and Ca- and/or Mg-free sea water treatment of the fertilized eggs to render them susceptible to re-fertilization. In all these experiments the re-inseminated eggs tend to become heavily polyspermic. Evidently the eggs have not only become re-fertilizable as a result of the treatment but also they appear to have lost the ability to propagate the normal rapid block to polyspermy characteristic of the untreated unfertilized egg (see Rothschild, 1954).

Eggs that are activated artificially can also be entered by a number of sperm if the membrane is removed, as in the early experiments of Loeb (1913) and Moore

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(1916, 1917) or especially if treated with Ca- and Mg-free sea water as in the recent experiments of Ishida and Nakano (1947, 1950) and Nakano (1954).

We have confirmed and extended some of the above experiments. In addition we have found that it is possible to re-fertilize eggs of the sea urchin *Lytechinus pictus* and *L. variegatus* at later stages without any treatment other than removal of the fertilization membrane.

MATERIALS AND METHODS

Eggs and sperm of the sea urchin *Lytechinus pictus* (Newport Bay, California) and *Lytechinus variegatus* (Alligator Harbor, Florida) were used in most of the experiments. Usually they were obtained by the method of KCl injection (Tyler, 1949). The sand dollar *Dendraster excentricus* was used in two sets of experiments, the eggs and sperm being obtained in the same way.

Mechanical demembranation was accomplished by means of a syringe and 20 gauge needle, the eggs being drawn through the needle at about two minutes after insemination when the fertilization membrane was almost fully elevated.

The trypsin employed in certain of the experiments was a crystalline preparation (Nutritional Biochemicals Corporation) and the papain was a non-crystalline preparation (Merck). The latter was prepared for use as described by Tyler and Spiegel (1956), 2 g. of the papain being extracted with 100 ml. 10^{-3} *M* Versene-sea water and filtered. One ml. of this is added to 100 ml. 0.2% L-cysteine hydrochloride (Merck) in 5×10^{-4} *M* Versene[5]-sea water and adjusted to pH 8.

The Ca- and Mg-free medium (see Tyler, 1953) consisted of a solution of 100 volumes of 0.55 *M* NaCl, 2.2 volumes of 0.55 *M* KCl, 10.3 volumes of 0.37 *M* Na_2SO_4 and ca. 0.6 volumes of 0.55 *M* $NaHCO_3$ to bring the solution to pH 8.

In all of the experiments, except where otherwise noted, the semen was initially diluted to 1% concentration or higher in 10^{-3} molar Versene in sea water and stored as such at the experimental temperature. The Versene was employed because of its ability to maintain the motility and fertilizing capacity of the sperm unchanged for prolonged periods (see Tyler, 1953). The experiments were all run at 17° C., except where otherwise noted.

RESULTS

Re-fertilization of mechanically demembranated eggs after treatment with Ca- and Mg-free sea water

The experiments of Sugiyama (1951) with Ca- and Mg-free sea water treatment could be readily confirmed with eggs of *Lytechinus*. We found, too, that it was not necessary to re-inseminate immediately after return of the eggs to normal sea water. If the demembranated eggs are given a five-minute treatment with Ca- and Mg-free sea water shortly after the first fertilization and returned to normal sea water they retain their ability to be re-fertilized for at least 30 minutes. The results of two experiments of this type are given in Table I.

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[5] Ethylenediamine tetraacetic acid. Acknowledgment is made to the Bersworth Chemical Corporation, Framingham, Mass. for a supply of this reagent.

TABLE I

Re-fertilization of eggs of Lytechinus pictus after mechanical demembranation and treatment with Ca- and Mg-free sea water for 5 minutes at 3 minutes after the first insemination

Time between first and second insemination	Percentage of polyspermy (0.1 ml. of 1% semen added per 5 ml. egg suspension)	
	Experiment 1	Experiment 2
Not re-inseminated	4	4
8 minutes	87	49
13 minutes	46	80
23 minutes	54	28
38 minutes	68	12

Re-fertilization of mechanically demembranated eggs without further treatment

As an additional control in the above-mentioned experiments the treatment with Ca- and Mg-free sea water was omitted and a high percentage of polyspermy was obtained upon re-insemination. A series of tests was therefore made of the re-fertilizability of eggs that had been subjected simply to demembranation and re-insemination. The results of five such experiments with eggs of *L. pictus* are listed in Table II. It is evident that fertilized and demembranated eggs can accept additional sperm as late as 40 minutes (at 17° C.) after the first insemination. The re-fertilizability is greater the shorter the time interval between the first and second inseminations, 100 per cent re-fertilization being readily obtained when the re-insemination is done within the first 10 minutes. Even at 15 minutes, as in experiment 5, the eggs can all be re-fertilized if sufficiently concentrated sperm is employed.

The data of the table also show that concentrations of sperm that give 100 per cent re-fertilization cause very little polyspermy when added as initial inseminates to the unfertilized eggs. It also appears from the data of Table II that the process of demembranation permits additional sperm of the initial inseminate to enter the eggs. This effect is, however, relatively slight as a comparison of the percentage polyspermy of the non-demembranated with that of the demembranated eggs in each of the experiments shows. Evidently most of the spermatozoa present in the initial inseminate are not capable of entering the demembranated eggs. Microscopic observation shows that a large proportion of the initially added sperm undergo the spontaneously reversing agglutination characteristic of the reaction with fertilizin in this species of sea urchins (*cf.* Tyler, 1948, 1955), and remain quite active thereafter.

Photographs of re-inseminated demembranated eggs are presented in Figure 1 along with those of non-demembranated and of demembranated and not re-inseminated, controls. In the sample re-inseminated at 10 minutes (Fig. 1e) all of the eggs are heavily polyspermic as shown by the clear areas representing the asters. The polyspermy here is too heavy to permit cleavage. In the various experiments, when the re-insemination is done early (10 to 16 minutes after the initial insemination) and with relatively large amounts of sperm (10^7 or more per ml. of egg suspension) practically all of the eggs show this high degree of polyspermy and failure of cleavage. From observations of the numbers of sperm asters it appears that five or more sperm can enter heavily re-inseminated eggs. In less heavily re-

TABLE II—*Re-fertilization of mechanically demembranated eggs of Lytechinus pictus at various times after fertilization*

Experiment No.	Time between first and second insemination (minutes)	Number of sperm ($\times 10^6$) per ml. of egg suspension		Percentage polyspermy determined at first cleavage (ca. 2 hours)
		First insemination	Second insemination	
1	Not demembranated	1.2	0	1
	Demembranated	1.2	0	3.5
	2½	1.2	1.2	53
	4½	1.2	1.2	51
	10	1.2	1.2	67
	20	1.2	1.2	20
	30	1.2	1.2	13
	40	1.2	1.2	35
2	Not demembranated	28	0	1
	Demembranated	28	0	5.6
	3	28	28	100
	5	28	28	100
	7	28	28	98
	10	28	28	96
	30	28	28	40
3	Not demembranated	2	0	2.5
	Demembranated	2	0	13
	5	2	6	100
	10	2	6	100
	20	2	6	81
	40	2	6	27
	Not616 demem. —40	2	0	6
4	Not demembranated	1	0	3
	Demembranated	1	0	22
	10	1	0.4	37
	10	1	1	51
	10	1	4	100
	10	1	8	100
	40	1	0.4	32
	40	1	1	35
	40	1	4	76
	40	1	8	65
5	Not demembranated	49	0	2
	Demembranated	49	0	3
	8	49	49	100
	8	49	245	100
	15	49	49	68
	15	49	245	100
	30	49	49	11
	30	49	245	78
	40	49	49	17
	40	49	245	61

In experiments 1 to 4 the semen was diluted initially to 1% in $10^{-3}M$ Versene in sea water and stored for the later inseminations at that concentration. In experiment 5 the semen was diluted to 10% in ordinary sea water. Sperm counts were made in experiments 2, 4 and 5; sperm estimated in experiments 1 and 3 from dilution on the basis of a content of 2×10^{10} per ml. semen.

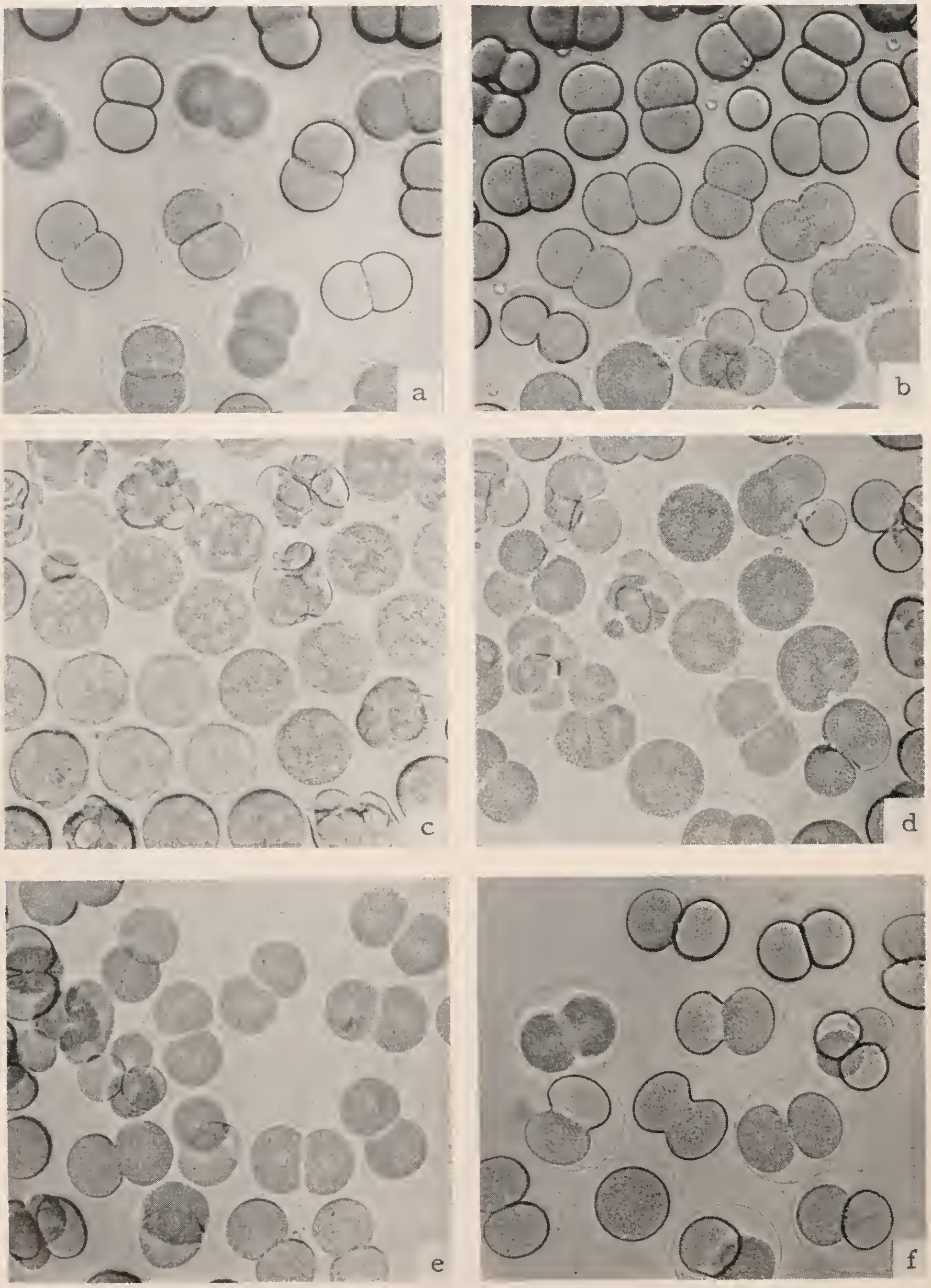

FIGURE 1. Re-fertilization of mechanically demembranated eggs of *Lytechinus pictus*. Insemination number 1 in 0.01%, number 2 in 0.03% semen. Eggs demembranated at one minute

inseminated, demembranated eggs, and in those re-inseminated at later times, the eggs that become re-fertilized generally accept fewer sperm and cleavage occurs in the form characteristic of dispermic or trispermic eggs (see Fig. 1d and e).

Two sets of experiments were performed with mechanically demembranated eggs of the sand dollar *Dendraster excentricus*. In one of these the re-inseminations at 10 and 30 minutes with 10^8 sperm per ml. gave 8.2 and 5.1 per cent polyspermy, respectively, as compared with 1.6 per cent in the non-reinseminated control. In the other experiment re-insemination with 2.2×10^7 sperm per ml. at 6 and 10 minutes gave 9.2 and 7.3 per cent polyspermy and 3.8 per cent in the controls. The results with lower concentrations of sperm and later times of re-insemination differed less significantly from the controls.

Experiments with mechanically demembranated eggs of *Lytechinus variegatus* gave results similar to those obtained with *L. pictus*. Two sets are presented in Table III which includes a comparison of eggs which had been subjected to Ca- and Mg-free sea water with eggs that were not so treated. In the first set the initial insemination was rather heavy (2% of semen which was originally diluted somewhat, perhaps ¼, by fluids shed with the semen). The non-demembranated, once-inseminated controls gave 10.5 per cent polyspermy. Demembranation alone at 1½ minutes resulted in an increase in polyspermy to 45 (Ca- and Mg-free sea water-treated eggs) and 58 per cent (untreated eggs), presumably due to re-fertilization by sperm present in the initial inseminate. Re-insemination at 30 minutes resulted in re-fertilization of most of the eggs when dense sperm suspensions (1% and 0.5%) were used and less with the more dilute suspensions. The treatment with Ca- and Mg-free sea water resulted in generally higher percentages of re-fertilization but the effect in this set is slight.

In the second set of experiments listed in Table III the initial insemination was performed with more dilute sperm and the control polyspermy was lower (7%). Demembranation without reinsemination resulted in a small increase in percentage polyspermy (17% for the Ca- and Mg-free sea water-treated eggs and 13% for the untreated eggs). Upon re-insemination at 36 minutes the large increase in polyspermy was again obtained when relatively dense sperm suspensions were used and significant increases even in the dilute suspensions. Again the Ca- and Mg-free sea water-treated eggs give somewhat more re-fertilization than the untreated eggs. In this set inseminations of normal, unfertilized, eggs were made, at the same time and with the same sperm suspensions that were employed for the re-insemination. These controls (not listed in the table) gave 100 per cent fertilization in all but the 0.0063 per cent concentration of sperm (semen) which gave 90 to 95 per cent. Versene was not used in these experiments on *L. variegatus*.

Re-fertilization of trypsin-treated eggs

When unfertilized sea urchin eggs are treated with trypsin they fail to elevate a membrane upon fertilization as Runnström *et al.* (1943) originally showed. Hagström and Hagström (1954a) found that such eggs could be re-fertilized if

after initial insemination. All pictures taken at time of first cleavage of control eggs. Magnification 95 ×. (a) Control, non-demembranated eggs. (b) Control, demembranated eggs. Re-inseminated (c) at 10 minutes, (d) at 20 minutes, (e) at 40 minutes after first insemination. (f) Control eggs, singly inseminated in 0.03% semen, at 40 minutes after beginning of experiment.

TABLE III

Re-fertilization of mechanically demembranated eggs of Lytechinus variegatus with and without treatment with Ca- and Mg-free sea water; at 22° C.

Concentration of sperm (semen)		Percentage polyspermy	
First insemination, %	Second insemination at 30 minutes	Eggs exposed to Ca- and Mg-free sea water at 6 to $12\frac{1}{2}$ minutes	Eggs washed in sea water at corresponding times
2	Not demembranated Not re-inseminated		10.5
2	Demembranated but not re-inseminated	45	58
2	1%	99.5	97
2	0.5	97.5	91
2	0.25	90	83.5
2	0.125	93	77.5
2	0.063	72.5	73.5
	Second insemination at 36 minutes	Ca- and Mg-free sea water at 5 to 11 minutes	
0.1	Not demembranated Not re-inseminated		7
0.1	Demembranated but not re-inseminated	17	13
0.1	0.1	74	86
0.1	0.05	77.5	56
0.1	0.025	60	52
0.1	0.0125	51	39
0.1	0.0063	44	40

TABLE IV

Re-fertilization of trypsin-treated eggs of Lytechinus pictus

First insemination; after 10 minute treatment of eggs with 0.01% trypsin in sea water and washing; Sperm per ml.	Second insemination		Percentage polyspermy
	Time of insemination in minutes	Sperm ($\times 10^6$) per ml.	
4×10^6	—	0	12
	5	8	47
	30	8	48
8×10^6	—	0	19
	5	8	73
	30	8	39
16×10^6	—	0	26
	5	8	76
	30	8	53

they were exposed to Ca- or Mg-free sea water for a short time at various times after the first fertilization. Practically no re-fertilization was obtained without the treatment with Ca- or Mg-free sea water.

We have found that when unfertilized eggs of *Lytechinus pictus* are exposed to trypsin, washed and inseminated, they can later be re-fertilized, without further treatment other than re-insemination. Table IV presents the results of a set of experiments in which the eggs were treated for ten minutes with a solution of 0.01 per cent trypsin in sea water. After washing, three samples were inseminated, each with a different concentration of sperm (4×10^6, 8×10^6 and 16×10^6 per ml.). Samples from each of these were re-inseminated at 5 minutes and at 30 minutes. Trypsin-treatment is known (see Hagström and Hagström, 1954b; Tyler and Metz, 1955) to render sea urchin eggs susceptible to polyspermy. In the present experiment this is evident in the relatively high percentage (12, 19 and 26) of polyspermy

TABLE V

Re-fertilization of papain-treated eggs of Lytechinus pictus

First insemination; eggs added to papain solution containing sperm; sperm per ml.	Time of papain treatment (minutes)	Second insemination (after washing eggs with sea water)		Percentage polyspermy
		Time in minutes	Sperm ($\times 10^6$) per ml.	
2×10^6	6	—	0	1
	6	14	2	2
	6	14	10	75
	6	40	2	2
	6	40	10	2
1.5×10^6	11	—	0	3
	11	18	1.5	10
	11	18	6	85
	11	40	1.5	8
	11	40	6	55
3×10^7	5	—	0	3
	5	7	30	95
	5	7	120	100
	10	—	0	5
	10	11	30	100
	10	11	120	100
	15	—	0	5
	15	17	30	98
	15	17	120	100
	20	—	0	18
	20	21	30	26
	20	21	120	100
	30	—	0	18
	30	32	30	100
	30	32	120	100
	40	—	0	17
	40	42	30	98
	40	42	120	100

obtained without re-insemination. However, the data of the table show that upon re-insemination re-fertilization occurs as late as 30 minutes after initial fertilization. The re-insemination at 5 minutes is relatively more effective.

Re-fertilization of papain-treated eggs

Treatment of unfertilized sea urchin eggs with papain also destroys their ability to elevate a membrane upon fertilization. The fertilizability of the papain-treated eggs is, however, often rather poor. Tyler and Spiegel (1956) showed that fertilization proceeds very well if the eggs are added to a suspension of sperm in the papain solution. In these circumstances a rather unique phenomenon is observed; namely, a fertilization membrane is elevated, as fully as in normal sea water and then, within the next three or four minutes, retracts to the surface of the egg from which it is not distinguishable. Such eggs, even when soon removed from the papain solution, show no signs of a fertilization membrane at the time of cleavage.

In the present experiments *Lytechinus* eggs were added to sperm in papain solution. At various times after retraction of the fertilization membrane to the surface the eggs were washed in sea water and re-inseminated. The results of three sets of experiments are presented in Table V. The insemination in the papain solution results in a relatively low (1 to 5) percentage of polyspermy except in those experiments (in the third set) where the eggs were allowed to remain in the solution for 20 minutes or longer in which case it rose somewhat (17 to 18%). Upon re-insemination one hundred per cent polyspermy was obtained in many of the tests. As in the experiments with the mechanically demembranated eggs and the trypsin-treated eggs the re-fertilizability is higher the sooner the second insemination is done and the greater the concentration of sperm. This statement applies to comparisons within a set of experiments since in experiments performed on different days the absolute values may differ, as a comparison of the first two sets of experiments in Table IV indicates. If the eggs are allowed to remain in the papain solution until shortly before the re-insemination, as in the third set of experiments listed in Table IV, the re-fertilizability at the later times remains high. As in the mechanically demembranated eggs the degree of polyspermy following re-insemination is often too high to permit cleavage, the polyspermic nature of the eggs being indicated by the many asters present.

DISCUSSION

It has been shown in the present experiments that fertilized eggs that have been removed from their fertilization membrane can be re-fertilized. This is contrary to the generally accepted views and the experience of many investigators (see Lillie, 1919; Just, 1939), the surface of the membrane-less fertilized egg being considered unreceptive to sperm. While the experiments of Tyler and Schultz (1932) demonstrated re-fertilizability of fertilized eggs this was confined to the period before membrane elevation occurs. The experiments of Sugiyama (1951) and of Hagström and Hagström (1954a, 1954b) showed that re-fertilization was possible at later stages, provided that the membrane-less eggs were exposed for a while to Ca- and/or Mg-free media. Without such treatment these workers obtained no significant re-fertilization of membrane-less eggs upon re-insemination. The difference in results may in part be due to differences in the species of echinoid used. In the present experiments the two preliminary experiments with *Dendraster* showed relatively little re-fertilizability in comparison with the results with *Lytechinus*. The difference may also be due in part to differences in the concentration of sperm used for re-insemination.

Sugiyama (1951) does not state the concentration of sperm used in his test (p. 336) of the re-fertilizability of denuded, untreated eggs of *Strongylocentrotus*. If it were 10^{-2} (one per cent semen; probably about 2×10^{-8} sperm per ml.), which, as shown in his Table I, was used for the eggs treated with Ca- and Mg-free sea water, this would be higher than concentrations found in the present experiments with *Lytechinus* to be capable of re-fertilizing practically all of the demembranated, untreated, eggs. Hagström and Hagström (1954a, 1954b) used 2×10^{6} sperm per ml. for re-inseminations of eggs of *Paracentrotus lividus* without getting significant re-fertilization of the controls. This is within the effective range for *Lytechinus* when the re-inseminations are performed early. It would appear, then, that species differences were largely responsible for the difference in results.

The use of Versene in the sperm suspension may have contributed to the effectiveness of the sperm in re-fertilization, by virtue of its favorable action (Tyler, 1953) on the fertilizing capacity of the sperm. However, it is not a determining factor in the present results, as the experiments (see Table II) in which it was omitted show. Also, it should be noted that in those experiments in which it was employed in the sperm suspension the amounts added with the sperm to the eggs were such as to make the concentration less than 10^{-5} molar.

One feature of *Lytechinus* eggs that might be related to the apparent ease with which they can be re-fertilized is the relative thinness, and slowness of formation, of the ectoplasmic (hyaline) layer. However, this layer is distinctly visible, and well formed, by about 15 minutes after fertilization, whereas re-fertilization is possible after 40 or more minutes.

It has been noted in the presentation of the data that the re-inseminated eggs tend to become highly polyspermic. This occurs with concentrations of sperm which, when used as a first inseminate, result in very little or no polyspermy. Evidently the demembranated fertilized egg does not possess the capability of the unfertilized egg of excluding all but one sperm. Experiments by Rothschild and Swann (1949, 1950, 1951, 1952; reviewed by Rothschild, 1954) have shown that the response of the unfertilized egg is characterized by a rapid change that passes over the surface in about two seconds, from the point of the entering sperm, that renders the surface about 95 per cent unreceptive to other sperm. This is followed by a change, requiring about 60 seconds, that makes the surface completely resistant. These changes are in advance of elevation of the fertilization membrane. However, the surface of the fertilization membrane is presumably this altered surface of the freshly fertilized egg. The surface of the denuded fertilized egg has evidently not become resistant to sperm and also lacks the ability to propagate a fast block to the entrance of more than one spermatozoon.

The ability of the denuded fertilized egg to be fertilized would imply that its surface possesses a sperm receptor substance similar to that of the unfertilized egg. In the latter this is attributed (see Tyler, 1948, 1955) to the substance known as fertilizin. It would be of interest to learn then whether the same kind of substance is present on the surface of the denuded unfertilized egg. Experiments by Motomura (1950, 1953a, 1953b) have provided some evidence for the existence of a cytofertilizin within the egg. The evidence also points to the ectoplasmic (hyaline) layer of the fertilized egg as being the likely source. If the results are not due to possible incomplete removal of the fertilizin of the unfertilized egg, they provide then an interpretation of the re-fertilizability of the fertilized egg on the same basis as that of initial fertilization; namely that the specific adherence of the sperm is due to reaction of its anti-fertilizin with fertilizin of the egg. If the fertilizin-like substance is assumed to be present in lower concentration on the surface of the denuded fertilized egg than on that of the unfertilized egg, or if it is assumed to be less reactive, this would help explain why high concentrations of sperm are required for re-fertilization. Further, it would appear that this postulated fertilizin-like substance is only slowly lost or altered on the surface of the denuded fertilized egg, the egg being re-fertilizable for a considerable period. Since brief treatment with Ca- and/or Mg-free media permits re-fertilization at still later stages and such treatment partially dissolves the ectoplasmic (hyaline) layer it would further appear that the treatment exposes additional fertilizin-like material. Tests of these and related possibilities will be the subject of future investigations.

SUMMARY

Fertilized, mechanically demembranated, eggs of the sea urchins *Lytechinus pictus* and *Lytechinus variegatus* can be re-fertilized when heavily re-inseminated at a half-hour or more (17° to 22° C.) after the initial fertilization. The re-fertilization occurs more readily the earlier the second insemination is performed. The re-inseminated eggs tend to become heavily polyspermic. Concentrations of sperm that give 100 per cent re-fertilization do not cause any significant polyspermy when used as a first inseminate on normal unfertilized eggs. The results show not only that the demembranated fertilized egg is capable of accepting additional sperm but also that it lacks the ability to propagate a fast block to all but one sperm. Treatment of the unfertilized eggs with trypsin, so as to suppress membrane formation, or with papain at the time of fertilization, so as to cause reversal of membrane elevation, also permits re-fertilization upon re-insemination without further treatment. The results are interpreted in terms of the possible presence of fertilizin-like sperm receptors on the surface of the denuded fertilized egg.

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ELEVATION AND RETRACTION OF THE FERTILIZATION MEMBRANE OF ECHINODERM EGGS FERTILIZED IN PAPAIN SOLUTIONS[1]

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It has been shown by Runnström *et al.*, (1943), Moore (1949) and others that treatment of unfertilized eggs of sea urchins with solutions of trypsin or chymotrypsin causes the eggs to fail to elevate a membrane upon fertilization. Normal development can ensue after such treatment, although the quantity of sperm required for successful fertilization is found (Tyler and Metz, 1956) to be greater than for untreated eggs. The treated eggs are also more susceptible to polyspermy (Hagström and Hagström, 1954) and more susceptible to cross-fertilization (Hultin, 1948; Bohus-Jensen, 1953; Tyler and Metz, 1956).

The action of trypsin in causing failure of membrane-elevation upon fertilization has been interpreted (Runnström *et al.*, 1943; Minganti, 1953) as due to a dissolution of the vitelline membrane of the unfertilized egg, which, together with material of exploded cortical granules, is considered (Motomura, 1941; Runnström *et al.*, 1944) to be the precursor of the fertilization membrane. Doubts have, however, been expressed (Mitchison and Swann, 1952; Hillier *et al.*, 1952) as to the separate existence of a vitelline membrane and of its dissolution by proteolytic enzymes (see also Tyler and Metz, 1956). The examination of the egg surface by polarization-microscopy (Swann and Mitchison, 1951; Mitchison and Swann, 1952) and by electron microscopy (Hillier *et al.*, 1952; see, however, McCulloch, 1952) revealed no distinct structure corresponding to the vitelline membrane. Runnström *et al.* (1943) showed that trypsin-treated eggs behave differently from untreated eggs upon shrinking and swelling in hypertonic and hypotonic solutions, which could be interpreted as due to loss of the vitelline membrane. It is possible, however, that the difference might be due to an alteration of the surface by combination with trypsin rather than the removal of anything therefrom. Parpart (personal communication) examined trypsin-treated eggs of *Arbacia* under the "television microscope" and could observe no change in the appearance of the vitelline membrane.

There are also cases, notably in the sand dollar *Dendraster excentricus* (Moore, 1951) and in the heart urchin *Echinocardium cordatum* (Runnström, 1948), in which treatment of the unfertilized egg with trypsin causes an elevation of a membrane.

In the present experiment the action of the enzyme papain was examined during the process of fertilization and it was observed that membrane elevation could occur

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in the presence of the enzyme but that the membrane subsequently returned to the surface of the egg.

Material and Methods

Eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* and of the starfish *Asterias forbesii* were used in this work.

The papain solution was prepared by extracting for several hours in the cold two grams of papain (Nutritional Biochemical Corporation) with 100 ml. of sea water containing 10^{-4} moles of Versene[3] (ethylenediaminetetraacetic acid). The Versene was included because of its effect of improving the fertilizing capacity of the sperm (Tyler, 1953). In some experiments the Versene was omitted. The extract (termed a 2% solution) was filtered and diluted (with 10^{-3} *M* Versene or with sea water) to the concentration desired. To this, just before use, cysteine hydrochloride was added to give a concentration of 0.2% and the pH adjusted to between 8.1 and 8.2.

Results

When unfertilized eggs of *Strongylocentrotus* or of *Lytechinus* were treated with solutions of papain ranging from 0.01 to 2% for periods of 5 seconds to one hour, and then inseminated in the solution, fertilization ensued without the elevation of a fertilization membrane. Such eggs cleaved while in the papain solution, the blastomeres separating from one another. When the unfertilized eggs were washed with sea water after treatment with papain solution (0.01% to 2% for periods of five seconds to one hour) they similarly failed to elevate a membrane upon fertilization. Cleavage of such eggs was normal and normal plutei developed.

When unfertilized eggs were added to a suspension of sperm in a 0.04% or 0.02% solution of papain, membrane elevation occurred. However, the membrane subsequently retracted to the surface of the egg. Figure 1 illustrates the course of this phenomenon in eggs of *Strongylocentrotus*. In this series eggs were added to a 0.1% suspension of semen in a 0.04% solution of papain. The membrane is observed to be fully raised after 1½ minutes (Fig. 1c). From then on it progressively shrinks until at 5½ minutes it has returned to the egg's surface, from which it is no longer distinguishable. If left in the solution the blastomeres tend to separate upon cleavage. If removed from the solution at 5½ to 20 minutes cleavage is normal with a distinct ectoplasmic (hyaline) layer visible, but no indication of a fertilization membrane (see Fig. 1, l). One hundred per cent normal cleavage can be obtained in this manner and later development is as good as in the control eggs.

Similar results were obtained with the eggs of *Lytechinus* and those of *Asterias*. In the latter the membrane elevates completely at 5 minutes and retracts to the surface (no longer distinctly visible) at 20–30 minutes after insemination.

Discussion

The main point of the present paper is that a fertilization membrane can be elevated and then retracted, as described above, during the first few minutes after insemination. There is no indication of the presence of this membrane as a dis-

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[3] Supplied by Bersworth Chemical Corporation, Framingham, Mass.

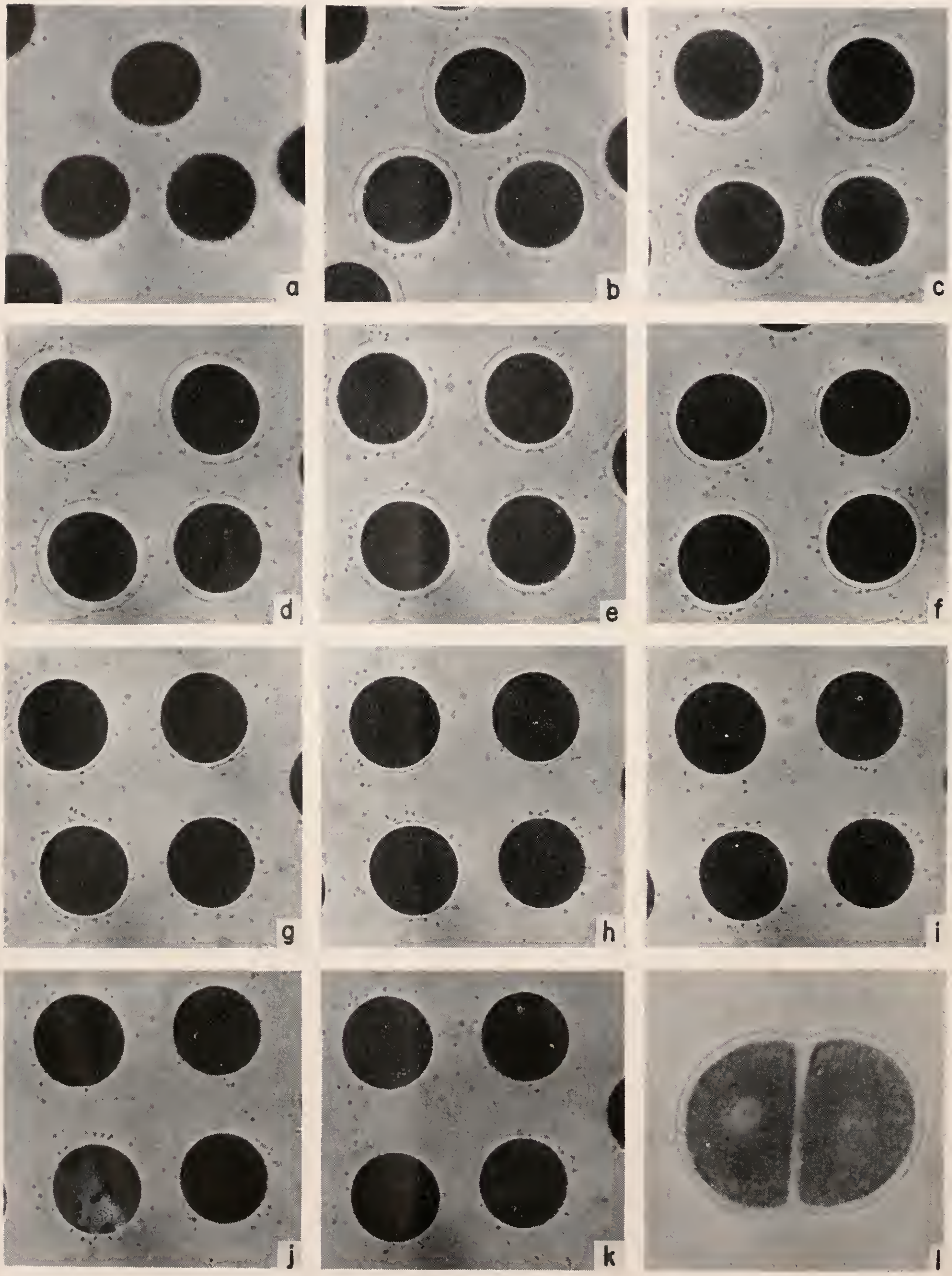

Figure 1. Papain demembranation of *Strongylocentrotus purpuratus* eggs. One drop sperm (in 0.02% papain) mixed with one drop egg suspension on slide sealed with vaseline. Figure (a) taken 35 seconds after eggs added, figures (b)–(k) taken at 30-second intervals. Figure 1 (l) shows a papain-treated egg (removed to sea water at 15 minutes) in the 2-cell stage. Magnification: Figures (a)–(k), 147 ×; figure (l), 333 ×.

tinct entity during subsequent development of the egg. This may be interpreted to mean that further enzyme-action after the membrane has reached the egg's surface results in its dissolution. It should be noted, however, that when eggs of sea urchins are treated with proteolytic enzymes after the fertilization membrane has fully formed (a few minutes after fertilization) the latter is not visibly affected (see Moore, 1949; Runnström *et al.*, 1943; Minganti, 1953). The same is true for treatment with papain solutions. This, then, does not support the interpretation that there has been a dissolution of the membrane by enzymatic action after return to the surface. Our present interpretation is that the contracted membrane fuses with the egg surface and its material remains a part thereof during subsequent development.

SUMMARY

When eggs of sea urchins and starfish are added to a suspension of sperm in papain solutions (0.02 to 0.04%), a fertilization membrane is elevated in the normal time and, after reaching full separation, retracts to the surface of the egg. This process is completed in approximately 5 minutes in the case of sea urchins (*Strongylocentrotus purpuratus* and *Lytechinus pictus*) and 25 minutes in the case of starfish (*Asterias forbesii*). It is not visible during subsequent development after removal of the eggs from the solution, and development to late stages is normal. Treatment of unfertilized eggs with papain prevents membrane elevation upon fertilization, as reported for other proteolytic enzymes. Treatment of eggs with fully formed fertilization membranes shows no effect on these. It is suggested that the retracted membrane, obtained by fertilization in papain solution, fuses with the egg surface, and that, in general, the question remains open as to whether or not proteolytic enzymes dissolve the vitelline membrane of the unfertilized egg.

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PHYSIOLOGY OF INSECT DIAPAUSE. X. AN ENDOCRINE MECHANISM FOR THE INFLUENCE OF TEMPERATURE ON THE DIAPAUSING PUPA OF THE CECROPIA SILKWORM

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Among the fifteen thousand Cecropia silkworms which we have reared during the past ten years, only an occasional individual has failed to begin a prolonged period of diapause immediately after pupation. Many of the animals underwent pupation in early summer, at the season when one might expect environmental temperature and photoperiod to favor uninterrupted growth and metamorphosis. Yet the intervention of diapause was no less uniform than in animals pupating in September or October. The Cecropia silkworm is therefore a typical single-brooded or "univoltine" insect.

The pupal diapause provides for the overwintering of the species. Though the pupa undergoes little or no morphological advance during this period, the months of exposure to the low temperatures of winter are not "time out." By its action within the diapausing insect, low temperature promotes certain physiological changes which assure the initiation of adult development the following spring. The detailed nature of these changes has been the central concern of the present study.

The investigation has been greatly assisted by a series of recent reviews surveying the voluminous literature pertaining to insect diapause (Andrewartha, 1952; Andrewartha and Birch, 1954; Lees, 1955). These reviews, to which the reader is referred for background to the present study, make clear that the potentiation of development by low temperature is a distinguishing feature of diapause, serving to differentiate diapause from hibernation, quiescence, and other forms of dormancy which are enforced by low temperature.

MATERIALS AND METHODS

The investigation was performed on pupae of the Cecropia silkworm (*Platysamia cecropia* L.). The larval stages were reared under large nylon nets on wild-cherry trees. The cocoons were harvested immediately after spinning and placed at 20 to 25° C. for at least six weeks prior to use. Pupation occurred during the first two weeks and was followed by the characteristic decline in metabolic rate to the extremely low diapausing level (Schneiderman and Williams, 1953).

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The temperature relations of several other species of diapausing saturniids have also been studied in detail. However, in the present report attention centers on the Cecropia silkworm, and only one series of experiments will be described on the related species, *Samia walkeri* (*cynthia*).

Ten years ago, at the outset of the present study, it was already evident that a quantitative approach to the problem required the use of a rather large number of individuals in testing each condition and circumstance. This practice, in turn, complicated the management of the experimental findings, since the latter sometimes showed considerable scatter. The procedure which was finally adopted and applied throughout the present study was to define the mean of each distribution, as well as the latter's standard deviation, by the use of probability paper (Codex Book Co., Norwood, Massachusetts, No. 32,451, Normal Ruling). The application of this technique, as well as various other experimental procedures, will be described in the sections that follow.

TABLE I

Previously unchilled Cecropia pupae at each of a series of constant temperatures: time for initiation of development and for adult development itself

Temp.	No. animals	Weeks to initiate development		Weeks to complete development		Total Weeks
		Mean ± S.D.	Recip. × 100	Mean ± S.D.	Recip. × 100	
2½° C.	20	∞	0	∞	0	∞
6	20	ca. 41	2.6	∞	0	∞
10	18	25.0 ± 1.9	4.0	16.3 ± 2.0*	6.1	41.3
15	19	23.3 ± 3.9	4.3	11.9 ± 1.4	8.4	35.2
20	20	37.0 ± 9.4	2.7	4.9 ± 0.6	20.4	41.9
25	20	25.4 ± 4.9	3.9	3.1 ± 0.9	32.3	28.5

* Adult moths with extremely dark pigment.

RESULTS

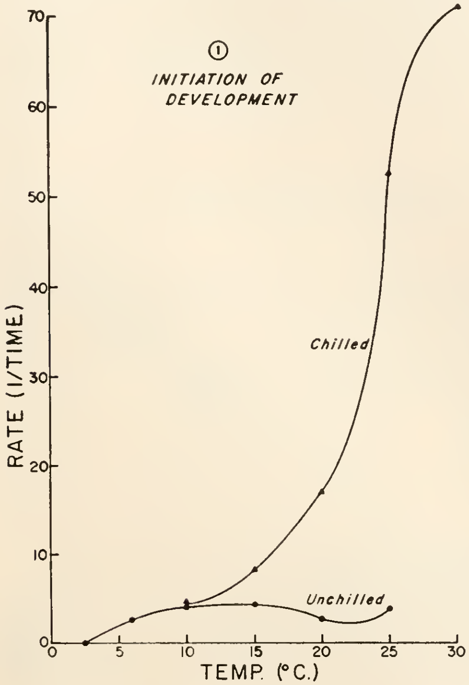
1. *Unchilled pupae at specific constant temperatures*

A. Initiation of adult development

When unchilled Cecropia pupae are placed at a particular temperature within the range 6 to 35° C. and maintained thereafter at this same constant temperature, the pupal diapause is ultimately terminated and adult development begins. In no case, however, does this occur immediately. Irrespective of what the temperature may be, adult development, as signalled by the retraction of the epidermis from the overlying pupal cuticle, is initiated only after prolonged exposure to the constant temperature. Prior to the actual initiation of adult development, even a detailed dissection of the pupa fails to reveal any clear-cut change from its condition at the outset.

In order to appraise the matter in quantitative terms, a study was made of the time required for the initiation of adult development at each of a series of constant temperatures. For this purpose, a homogeneous batch of animals (which had pupated 6 to 8 weeks earlier and has subsequently been stored at 20–25° C.) was removed from cocoons, placed in individual capped "creamers," and distributed among seven temperature cabinets or constant temperature rooms at temperatures ranging from 2½ to 30° C. Thereafter at weekly intervals, each pupa was checked for the initiation of adult development, as judged by the retraction of the leg epidermis.

In the experimental results summarized in Table I, it will be observed that a considerable period was required for the initiation of adult development at all of the constant temperatures within the range 2½ to 30° C. Indeed, at 2½° C. adult development never began, and pupae at this temperature invariably died after about a year.



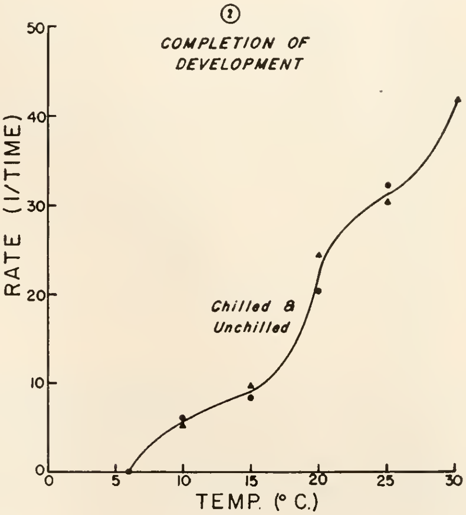


FIGURE 1. The rate (100/Weeks) of initiation of adult development at each of a series of constant temperatures. The lower curve describes the response of previously unchilled pupae; the upper curve, the response of pupae which had been chilled for 15 weeks at 6° C. prior to the experiment. The period of preliminary chilling greatly accelerates the initiation of development.

FIGURE 2. The rate (100/Weeks) of adult development at a series of constant temperatures after development has begun. The response of previously unchilled pupae (circles) cannot be distinguished from that of previously chilled pupae (triangles).

In the lower curve in Figure 1 the results have been computed and plotted on a rate (1/Time) basis. The relatively minor effects of temperature are here clearly evident. Even these minor effects are paradoxical in that the rates encountered at 20° and 25° C. are actually lower than those at 10° and 15° C.

B. Completion of adult development

Each animal in the experiment just considered was permitted to continue to develop and to emerge as an adult moth. In this manner it was possible to ascertain the effects of temperature on the kinetics of adult development itself. The results are recorded in Table I, and plotted on a rate (1/Time) basis in Figure 2.

It is clear that once adult development has begun, a striking change occurs in the insect's response to environmental temperature. Temperature now becomes a prime determinant of the rate of adult development—a fact which is signalled in Figure 2 by the curve's pronounced and positive slope.

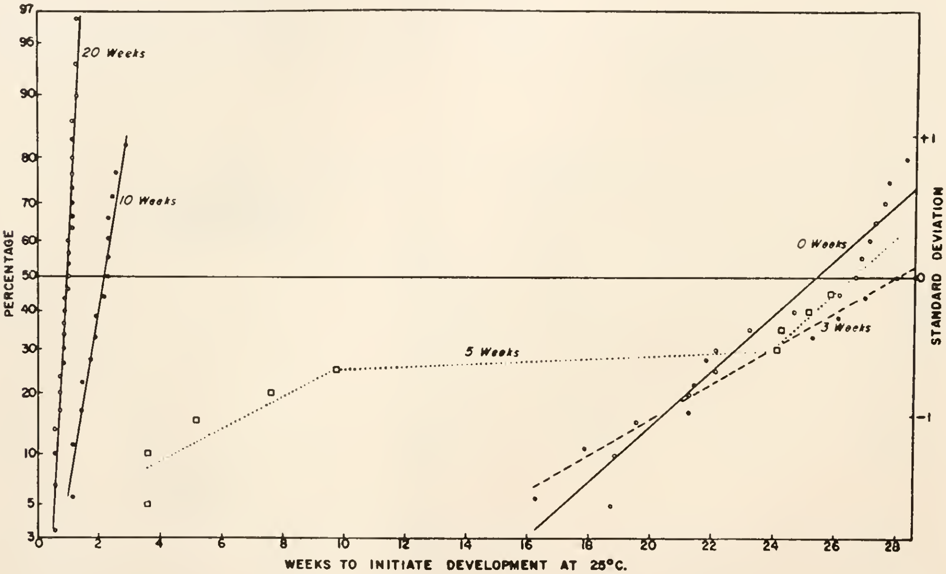


FIGURE 3. Time required for the initiation of adult development at 25° C. after preliminary exposure to 6° C. for 0–20 weeks. By the use of a probit scale on the Y-axis, each distribution is converted into a straight line. The discontinuous line for the 5-week series signals a bimodal distribution.

Though the formation of the adult moth, as noted in the preceding section, ultimately begins after about ten months at 6° C., such animals never proceed beyond early development and ultimately die before reaching a stage corresponding to that which would be attained at 25° C. after only three days. At the slightly higher temperature of 10° C., approximately 3½ months elapse between the initiation and completion of development. Adult moths developing at this constant temperature are extremely dark, the reddish pigments of scales and hairs being replaced by black and the white pigments by gray. At 10° C. the insect is incapable of sufficient muscular effort to emerge spontaneously from the pupal exuviae and ordinarily does not do so unless returned to higher temperatures. By contrast, at 15° C. and all higher temperatures up to 30° C., the moths show normal pigmentation and are able to emerge spontaneously and spread their wings. At the still higher temperature of 35° C., development proceeds with considerable rapidity, but the moths are extremely pale and feeble, and female individuals show few if any eggs.

2. *Previously chilled pupae at specific constant temperatures*

The experiment described in the preceding section was repeated on a homogeneous batch of pupae which had been previously exposed to 6° C. for 15 weeks. The results are summarized in Table II and plotted on a rate (1/Time) basis in Figures 1 and 2.

The upper curve in Figure 1 makes clear the extraordinary difference in the temperature response of chilled pupae from that previously defined for unchilled pupae (lower curve). Temperatures above 10° C. now greatly accelerate the initiation of adult development. For example, at 25° C. the time required for the initiation of adult development is decreased from 25 weeks to 13 days.

TABLE II

Previously chilled pupae (6° C. for 15 weeks) at each of a series of constant temperatures: time for initiation of development and for adult development itself

Temp.	No. animals	Weeks to initiate development*		Weeks to complete development		Total Weeks
		Mean ± S.D.	Recip. × 100	Mean ± S.D.	Recip. × 100	
10° C.	8	21.4 ± 7.4	4.7	19.1 ± 4.3	5.2	40.5
15	19	12.1 ± 14.1	8.3	10.3 ± 0.9	9.7	22.4
20	19	5.9 ± 4.7	17.0	4.1 ± 1.9	24.4	10.0
25	30	1.9 ± 1.0	52.7	3.3 ± 0.3	30.3	5.2
30	19	1.4 ± 1.0	71.4	2.4 ± 0.3	41.7	3.8

* Not counting the previous 15 weeks at 6° C.

Attention is directed to the tempo of adult development after the latter has begun (Fig. 2). Manifestly, the curve descriptive of the response of previously chilled pupae cannot be distinguished from that recorded for unchilled pupae. We are therefore led to infer that the pupal diapause, as a physiological and biochemical phenomenon, is primarily concerned with events which culminate in the initiation of development, and that it is little concerned with adult development itself. Our present problem, in brief, is to account for the differences between the upper and lower curves in Figure 1.

One can say immediately that the upper curve, which describes the response of previously chilled pupae, is not remarkable: it is a familiar experience to find biological processes accelerated by temperature. The previously chilled pupa, in effect, is responding to temperature like a normal non-diapausing insect. But what is remarkable is the effective "insulation" of the previously unchilled pupa from the accelerating action of temperature. An explanation of this effect was the primary objective of the present study.

3. *Graded exposure of unchilled pupae to 6° C.*

We have already considered and illustrated in Figure 1 the way in which preliminary exposure to 6° C. greatly decreases the time required for the initiation of development at 25° C. The speed with which the response ensues at 25° C. becomes a measure of the persistent effects of the previous exposure to low temperature. In the experiment recorded in Figure 1, the exposure to 6° C. was for 15 weeks. However, the same technique obviously can be used to appraise the quantitative effects of any other duration of chilling. This maneuver has been used extensively during the present study in order to probe the latent effects of low temperature.

Figure 3 summarizes a part of the results of a detailed study of this type in which the effects of the duration of exposure to 6° C. were studied. The experiment was performed on 160 Cecropia pupae which were harvested in freshly spun cocoons on August 7–11, 1953, and temporarily stored at 25° C. for ten weeks prior to the experiment. The cocoons were then placed in capped cardboard containers and stored at 6° C. Thereafter, at regular intervals ranging from 0 to 45 weeks a group of twenty pupae were removed from cocoons, placed in individual, uncapped "creamers," and returned to 25° C. The time which each animal required to initiate adult development was then ascertained in terms of the initiation of retraction of the leg epithelium—a signal of the second day of adult development at 25° C.

The effects of the graded exposures to 6° C. are plotted in Figure 3 on a probability grid, whose use was mentioned in the section on Methods. This type of plot has the advantage of transforming normal distributions into straight lines; it likewise permits one to define the mean and the standard deviation with far greater precision than is possible by any simple arithmetic procedure. In Figure 3 a straight line has been drawn by inspection through the distribution of responses after each preliminary exposure to 6° C. It is immediately apparent that 3 weeks at 6° C. failed to facilitate the subsequent response at 25° C.; indeed, the mean (28 weeks) was slightly prolonged beyond the value after no chilling (25.4 weeks); moreover, as signalled by the flatter slope to the curve, the scatter in the data (which was already large after no chilling) was further enhanced after three weeks at 6° C.

In Figure 3 attention is now directed to the distribution of responses at 25° C. after preliminary exposure to 6° C. for 10 and 20 weeks, respectively. It is clear that all these data fall nicely into straight lines whose steep slopes signify minor degrees of scatter. It is of particular interest and importance to note that the means, thus defined, are extremely small and range from 7 to 15 days. Consequently, a spectacular effect of the low temperature treatment has taken place between the third and tenth weeks at 6° C.

Within this critical period the response was examined after 5 weeks of chilling. In the results recorded in Figure 3, it is necessary to place a discontinuous line through the data thus obtained. This signifies that the distribution of responses is bimodal. Five of the twenty pupae, irrespective of sex, showed a significant effect of the low temperature treatment. An extrapolation to the 50 per cent level of a line joining these five points suggests a mean value of approximately 16 weeks for the initiation of development. But for the next five animals to initiate development, it is clear that 5 weeks at 6° C. was without detectable effect, the estimated mean being, in fact, slightly greater than that observed after no chilling.

The response after 5 weeks of chilling is therefore of particular interest in that 25 per cent of the pupae showed a significant influence of the preliminary low temperature treatment while 75 per cent did not. Since the response after 10 weeks of chilling is homogeneous, it follows that in the normal course of events, this residual 75 per cent must shift from the delayed to the accelerated type of response between the fifth and tenth weeks at 6° C.

A point of considerable theoretical interest is the lack of overlap between any regions of the 5-week and 10-week curves in Figure 3. This signifies that even in the five animals which responded most promptly in the 5-week series, the physiological changes responsible for the accelerated response had not proceeded to completion by the fifth week, but, so to say, were caught "in transit."

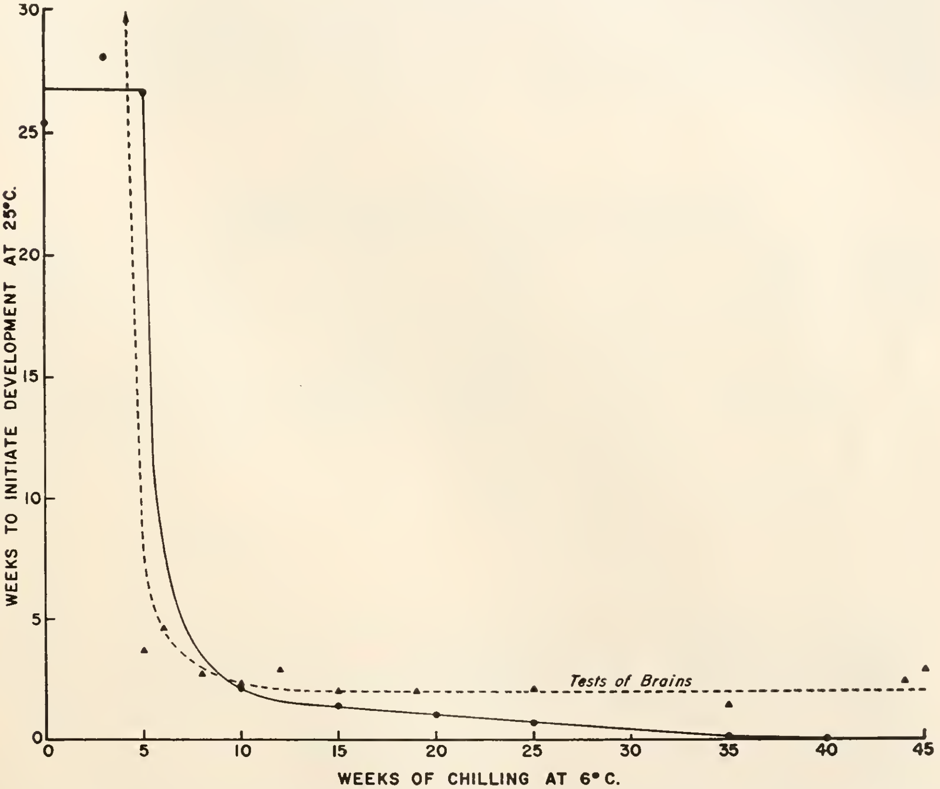


FIGURE 4. The unbroken curve records the time required for the initiation of development at 25° C. as a function of the duration of preliminary exposure to 6° C. The broken line records the corresponding changes occurring in the endocrine activity of the pupal brain. For explanation, see text.

On the basis of the results just considered, one can therefore recognize a sequence of two relatively stable states during pupal life, corresponding, respectively, to the *delayed* and the *accelerated* responses to the warm temperature. Moreover, the transition from the first to the second state shows the kinetics of a threshold reaction, being initiated by each individual during an apparently brief period after the third week at 6° C. and prior to the tenth week. It is equally clear that this reaction, which then effects the transition, can scarcely be "all or none," since it requires at least several weeks to pursue its full course.

These various conclusions are illustrated in Figure 4 where the mean times required for the initiation of development at 25° C. have been read from Figure 3 and plotted as a function of the duration of preliminary chilling at 6° C. The abrupt descending limb of the curve has been placed just after the fifth week at 6° C., though, for reasons mentioned above, its true position for about one-fourth of individuals is slightly to the left of this point.

Notwithstanding persistent uncertainties with respect to the precise time-course of the descending segment of the curve, there can be little doubt that the relationship is dominated by the transition of the pupa from a state of delayed response at 25° C. to one of accelerated response. This transition, we have inferred, is complete by the tenth week at 6° C. Yet it will be observed that after the tenth week of chilling a slow downward trend continues in the lower horizontal limb of the curve in Figure 4. Thus, after 35 weeks at the low temperature, adult development begins after only one day at 25° C. And after approximately 40 weeks, adult development actually begins at 6° C. It is equally clear, however, that the changes after the tenth week are extremely slow compared to those occurring during the first ten weeks at 6° C. Moreover, there are substantial reasons, to be considered below, for separating the early and the late effects and attributing the gentle slope of the lower horizontal part of the curve to secondary changes within the fully "accelerated" pupa.

TABLE III

Time for initiation of development of Cecropia pupae at 25° C. after indicated exposures to specific constant temperatures*

Exposure (weeks)	2½° C. (weeks)	6° C. (weeks)	10° C. (weeks)	15° C. (weeks)	20° C. (weeks)	30° C. (weeks)
0	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9	25.4 ± 4.9
3	—	28.1 ± 7.6	24.6 ± 4.7	—	—	—
5	28.0 ± 10.6	26.7**	20.9**	16.0**	23.7 ± 12.9	22.6 ± 2.7
10	26.3 ± 15.0	2.1 ± 0.7	1.7 ± 0.3	2.0 ± 0.4	5.3**	25.0 ± 20.0
15	2.1**	1.4 ± 0.7	1.0 ± 0.4	1.1 ± 0.6	3.4 ± 1.9	26.0 ± 19.1
20	—	1.0 ± 0.3	—	—	—	—
23	—	—	—	Zero***	—	—
25	0.6 ± 0.3	0.7 ± 0.3	Zero***	—	—	—
35	—	0.1 ± 0.1	—	—	—	—
37	—	—	—	—	Zero***	—
40	—	Zero***	—	—	—	—

* Each datum is the mean ± standard deviation (in weeks) of 18–20 pupae, and was derived from a plot of the individual responses on probit paper.

** Bimodal distributions. The mean characteristic of the majority of the sample is recorded.

*** Development is initiated under conditions of constant exposure to the temperatures noted.

4. *Potentiation of development by temperatures other than 6° C.*

We have considered up to this point the potentiation of development by prior exposure to 6° C. The same technique permits one to examine the latent effects of any other temperature within the physiological range. The temperatures selected for study in this manner were 2½, 10, 15, 20 and 30° C., the effects in each case being judged in terms of the time subsequently required for the initiation of adult development at 25° C. In order to facilitate comparisons with the results at 6° C., unchilled diapausing pupae of the same 1953-brood were utilized and all experiments were set up synchronously. The over-all results, including those previously described for 6° C., have been summarized in Table III.

In Figure 5 the data have been computed on a rate (1/Time) basis and plotted as a series of asymmetric, bell-shaped curves. Each curve records the rate with which adult development is initiated at 25° C. after a particular period of preliminary exposure to temperatures ranging from 2½ to 30° C. For comparison, the corresponding rate in the absence of any preliminary treatment has been recorded as the straight line paralleling the X-axis.

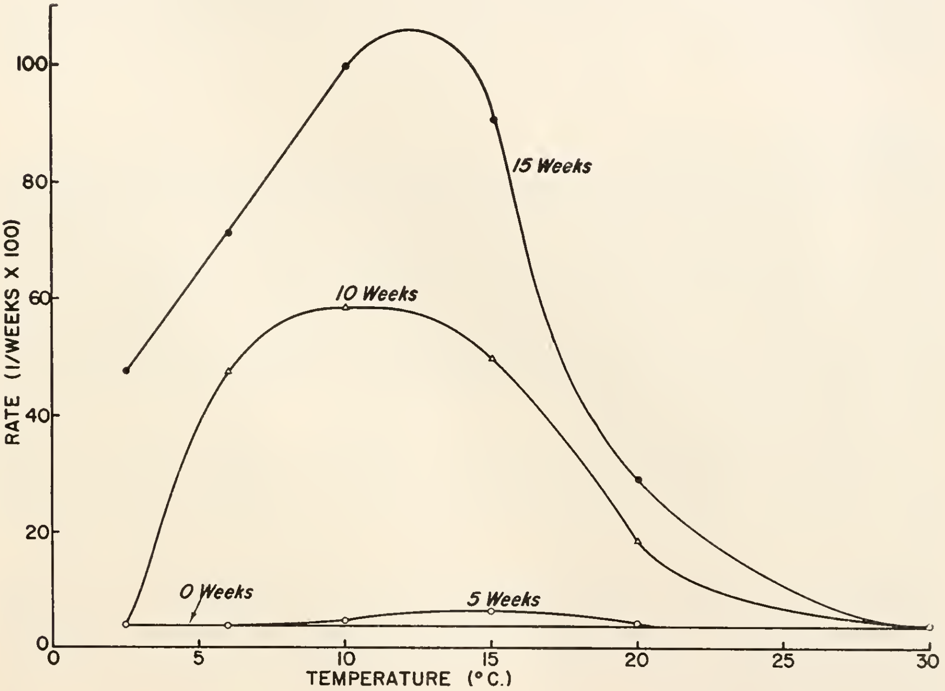


FIGURE 5. The rate (100/Weeks) of initiation of adult development at 25° C. after preliminary exposure to 2½–30° C. for 0–15 weeks.

Exposure to any of these temperatures for less than 5 weeks is without detectable effects on the subsequent rate of development at 25° C.—a conclusion already noted in the experiments at 6° C. After 5 weeks of the preliminary treatment, the distribution of results becomes bimodal at 6, 10, and 15° C. (see Table III). In Figure 5 this fact is not evident in the 5-week curve because the plotted points describe the primary mode in each of the three bimodal distributions. However, the bimodality is of interest in itself because it signals the onset of a period of rapid change.

This inference is confirmed in the results recorded after 10 weeks of preliminary treatment. Here a pronounced potentiation of development is apparent after exposure to 6–15° C. And, here also, the results at 20° C. become bimodal (Table III). A continuation of this trend is evident after 15 weeks of exposure, when, for the first time, the range of effective temperatures is broadened to include 2½° C. And, finally, as recorded in Table III, adult development actually begins by the 25th week of continuous exposure to 10–15° C.

The results as a whole are therefore in general agreement with the analysis suggested in connection with the experiments at 6° C. It is of particular interest that an intermediate range of temperatures (6 to 15° C.) is far more effective in potentiating development than are temperatures lower than 6° C. or higher than 15° C.

5. *Effects of brain removal*

The analysis, up to this point, has focussed attention on a physiological change within the diapausing insect which mediates the catalytic effects of low temperature. As long as ten years ago there were already substantial reasons for localizing the site of this physiological change in the pupal brain. It was found that low temperature alters the pupal brain in such a manner that the brain regains its competency to secrete a hormone prerequisite for the initiation of adult development (Williams, 1946). It was also possible to show that the secretion of this hormone is necessary until adult development actually begins. Consequently, if one removes the pupal brain prior to the initiation of development the pupa is stabilized in a state of "permanent diapause."

Manifestly, these previous findings have an intimate bearing on the results of the present study. If the action of low temperature is mediated solely by the brain, then it should be possible to "excise" the effects of prior chilling by removing the brain.

Experiments of this type have been performed on a considerable scale during the past ten years. The results can be summarized most briefly in the statement that among several thousand chilled pupae from which the brains were removed prior to the initiation of development, not a single individual escaped from diapause and initiated development.

Consider, for example, pupae chilled for 35 weeks at 6° C. Though such animals, as illustrated in Table III, initiate development after only one day at 25° C., development is permanently blocked if the brain is removed during those first 24 hours. Such brainless animals continue to live at 25° C. for up to two years thereafter, and during this period cannot be distinguished morphologically from brainless unchilled pupae. By this and analogous procedures it was possible to confirm the fact that the effects of prior chilling can be eliminated by excision of the brain.

Whereas it was initially thought that the brain is no longer necessary after the earliest indication of adult development (Williams, 1946), more detailed studies showed that the brain continues to be required during the first 24 hours of adult development, and, indeed in rare individuals, during the first 48 hours (Williams, 1952). It is of particular interest that excision of the brain during the first 24 hours blocks the further progress of development. Dissection of such animals showed that the epidermis (which had already loosened its attachments to the pupal cuticle and begun to retract) re-appressed itself to the cuticle and re-established its extremely intimate connections. In effect, therefore, the insect over a period of several weeks returns to a state indistinguishable by any morphological sign from that of a diapausing pupa.

An occasional animal from which the brain had been removed during the second day of development showed an anomalous response in that development of certain specific regions, including head, thorax, wings, legs, and antennae, continued for a time and then stopped with these parts in a more or less advanced stage of adult differentiation. But in virtually all cases where brain removal was postponed until after the first 48 hours of adult development, the latter continued to yield brainless but otherwise normal moths.

6. *Quantitative changes in brain activity during chilling*

The experiments just considered point to the pupal brain as the vehicle by which low temperature catalyzes the termination of diapause. Under this circumstance one would anticipate systematic changes in the brain's endocrine activity to account for the quantitative aspects of the low temperature effect; *i.e.,* for such changes in development potential as one observes in Figures 4 and 5.

The most detailed study of this sort was performed in 1953–1954 in synchrony with the above-mentioned temperature studies and on aliquot samples of the same homogeneous group of animals. At the outset of the experiment the brains were removed from ten unchilled individuals and tested for activity by implanting them under a plastic window at the tip of the abdomen of a corresponding number of brainless unchilled pupae. Subsequently, at intervals of one to several weeks, the brains of additional 6° C.—animals were removed and tested in like manner. By this procedure it was possible to quantitate the endocrine activity of each implant in terms of the time which it required to evoke the development of the host at 25° C.

The results are summarized in Table IV. It is evident that only an occasional brain was active when obtained from pupae chilled for less than 5 weeks; moreover, in such instances, development ordinarily began only after a considerable delay (up to 40 weeks). By contrast, brains chilled for 5 weeks or longer were not only active in a high proportion of cases, but also, with rare exception, evoked the initiation of adult development within a month after implantation. Evidently, on or about the eighth week at 6° C., the brain attains maximal activity, as judged by the high percentage and uniform rapidity of the responses. Presumably, the occasional inactivity encountered after the eighth week was caused by injury to the brain during the latter's excision and transplantation.

It is particularly fruitful to compare the change in the brain's activity with the previously described behavior of intact pupae after systematic exposure to 6° C. To this end, the mean values in Table IV have been plotted as the broken-line curve in Figure 4. The degree of congruity between these curves leaves little doubt that the abrupt shift in the developmental potential of the pupa as a whole is the outcome of an equally abrupt shift in the endocrine activity of the brain.

Notwithstanding the substantial correlation between the two curves in Figure 4, one also observes minor but highly significant differences. Thus, after chilling for less than 5 weeks, only about one brain in three was found active when tested by implantation. Yet, even in the absence of chilling, the undisturbed brains of normal pupae exert their effects during the first year at 25° C. This is one of the few differences encountered between the endocrine activity of "loose" implanted brains and brains retaining normal nerve connections. However, such connections are clearly not obligatory since briefly chilled implants were able to achieve threshold activity in one-third of the cases.

A second difference between the curves in Figure 4 is the fact that 50 to 70 per cent of excised brains showed full activity when tested after 5 or 6 weeks of chilling, whereas only about 25 per cent of unoperated pupae developed promptly after the same amount of chilling. Since this period coincides with the time of rapid change in brain activity, conceivably the difference is a sampling artifact attributable to the limited number of animals tested. However, it is even more likely that the difference is real and that, by some unknown means, the surgical manipulation of the brain during this critical period hastens to completion the changes which restore the brain's endocrine activity.

Table IV

Tests of endocrine activity of brains from pupae previously chilled at 6° C. for specific periods*

Prior chilling of brains (weeks)	Number tested	Number active	Time** to evoke development at 25° C. (weeks)
0	10	2	10; 11; – —
3	10	4	3.3; 3.3; 23.9; 40.6; —
4	10	3	2.7; 3.0; 37.9; —
5	20	14	3.7 ± 1.1
6	10	5	4.6 ± 1.9
8	10	7	2.7 ± 1.3
10	10	10	2.3 ± 0.6
12	10	7	2.9 ± 1.1
15	10	10	2.0 ± 0.9
19	16	16	2.0 ± 0.7
25	8	8	2.1 ± 0.6
35	10	9	1.4 ± 0.3
44	9	8	2.4 ± 1.7
45	22	19	2.9 ± 1.1

* Each brain was implanted into a brainless diapausing pupa and the time for the latter to initiate adult development at 25° C. determined.

** Mean ± standard deviation in weeks are recorded for all groups in which at least 50 per cent of animals developed.

A final difference between the two curves in Figure 4 concerns the lower segments after the tenth week of chilling. It is clear that after 8–10 weeks at 6° C. the brain has fully regained its endocrine powers and that only minor changes occur thereafter. By contrast, in normal unoperated pupae the onset of development at 25° C. is progressively accelerated when the low temperature treatment is prolonged beyond the tenth week; indeed, we have already observed that adult development actually begins at 6° C. after about 40 weeks. This implies that a slow release of the brain hormone takes place at the low temperature and that a threshold titer is ultimately built up after about 40 weeks at 6° C. Consequently, after 35 weeks at 6° C. the titer is barely sub-threshold and further brain function at 25° C. is required for only one day. Since the excised brains were tested in brainless unchilled pupae lacking any substantial titer of brain hormone, one would anticipate the observed difference between the lower segments of the curves in Figure 4.

On the basis of these various observations, the effects of low temperature on the intact diapausing insect are subject to interpretation. Low temperature promotes a transformation of the brain so that the latter becomes competent to secrete its hormone; as illustrated in Figure 4, this process is complete by the tenth week at 6° C. The slow secretion of the hormone then begins at the low temperature. Once the brain has regained its endocrine powers, then, as illustrated in Figure 1, the secretion of the hormone is actually retarded by low temperature. The catalytic effects of low temperature on the diapausing pupa may therefore be said to end as soon as low temperature has induced the physiological transformation of the brain.

7. *In vivo and in vitro chilling of the brain*

In the course of the routine preparation of several thousand brainless diapausing pupae used in the studies of the present series, the excised unchilled brains were often implanted into brainless diapausing hosts and then subjected to chilling under conditions of *in vivo* culture. In this manner it was possible to build up a "brain bank" which was useful for various purposes. Such brains, after a suitable period of chilling, could then be reclaimed and utilized as active chilled brains.

This finding, in itself, gives assurance that the normal connections of the brain with the rest of the nervous system are not prerequisite for the temperature effect. However, as Andrewartha (1952) has pointed out, the experimental method did not exclude the possibility that low temperature acts indirectly on the brain—perhaps by making available within the host some precursor of the brain hormone.

Under this latter circumstance one would expect the hypothetical activating substance to be present in high concentration in pupae chilled for a prolonged period. This prospect was tested by excising the brains of previously chilled pupae and reimplanting one or more brains of unchilled pupae. In no case did the previously chilled host have any detectable effect in activating the unchilled implant. Manifestly, this result argues against the view that low temperature acts by promoting the release of some brain-stimulating factor within the pupa as a whole. It also opposes an alternative possibility that low temperature destroys a brain-inhibitor within the pupa as a whole.

A conclusive test of the direct action of low temperature on the brain obviously requires that the brain be activated apart from the rest of the insect; *i.e.*, under conditions of *in vitro* culture. This objective is not easy to realize in view of the unsatisfactory state of knowledge concerning the culture of insect tissues other than blood cells and spermatocytes (Schmidt and Williams, 1953).

Nevertheless, during the past ten years a considerable number of unchilled brains have been subjected to *in vitro* culture under sterile conditions, the media consisting of various types of physiological solutions or of blood obtained from chilled, unchilled, or developing animals. The blood-containing media invariably underwent darkening during prolonged exposure to low temperature, notwithstanding the presence of crystals of the potent anti-tyrosinase, phenylthiourea. Darkened blood is known to be extremely toxic to insect tissues, and such experiments were doomed to failure.

In no case did the brains survive the low temperature culture for longer than about two weeks. Such brains, when implanted into brainless pupae, were immediately attacked by phagocytic cells and ultimately broken down.

One might suppose that even dead brains, if sufficiently numerous, might contribute a critical concentration of hormone after implantation. This possibility was discounted by the finding that active brains, killed by freezing and thawing in liquid nitrogen or by exposure to temperatures higher than 50° C., were ineffective when implanted in large numbers. Evidently, only a trace of hormone is stored in the normal active brain, since the biological effect of a single hemisphere of a living brain cannot be duplicated by the implantation of fifty dead brains.

Consequently, for reasons that are believed to be technical in character, it has been impossible to accomplish this final and most direct test of the view that low temperature achieves its catalytic effect by its direct action on the brain. However, the sum total of available evidence gives little reason to think otherwise.

8. *Winter sickness*

It will be recalled that the onset of adult development under conditions of constant exposure to 6° C. is delayed until approximately the fortieth week at the low temperature. Even then, the development which takes place fails to proceed beyond an early stage, corresponding to that attained after only two days of adult development at 25° C. If the exposure to 6° C. is continued still longer, one detects the onset of a curious syndrome for which we suggest the name "winter sickness." The distinctive feature is a progressive paralysis of the intersegmental muscles of the abdomen; *i.e.,* the muscles responsible for the "tone" of the motile abdominal segments and the latters' characteristic circular motion in response to mechanical stimulation.

In pupae returned to room temperature after 46 weeks at 6° C., the tone of these muscles is subnormal for several days thereafter, and contraction can be evoked only when the pupa is subjected to mechanical stimulation such as squeezing. Further prolongation of the low temperature treatment leads to a flaccid paralysis of all muscles save the heart. As summarized in Table V, electrical excitability likewise disappears, and contraction can no longer be induced in response to tetanizing shocks administered to the abdomen by overlying electrodes.

The early stages of winter sickness are reversible at room temperature until about the fifteenth month at 6° C. (see Table V). After several days at 25° C., electrical excitability returns, followed shortly therafter by motion in response to mechanical stimulation, and, finally, by the recovery of tone. Adult development then continues where it had left off. However, if the exposure to 6° C. has been for longer than a year, the adult moths, when formed, are generally feeble and unable to spread their wings, or, indeed, to escape from the pupal exuviae. And in the case of female individuals only a few eggs are matured.

After 15 months at 6° C., winter sickness becomes irreversible for a progressively larger number of individuals. Such animals show the absence of heart beat when returned to room temperature, and, in fact, are already dead, as judged by the lack of any detectable oxygen consumption.

Winter sickness is not peculiar to the Cecropia silkworm, since it has been regularly encountered in all species of diapausing pupae which we have studied at low temperatures. For example, *Telea polyphemus* shows reversible sickness after 60 weeks at 6° C. and death after 90 weeks; *Samia walkeri* (*cynthia*) shows flaccidity after 35 weeks at 6° C. and death after 90 weeks. *Antheraea mylitta* and *Actias selene* are even more susceptible, being killed by 6 months at 6° C.

A considerable number of experiments were performed in search of the physiological basis of winter sickness. Of particular interest was the paralysis of the abdominal muscles—a condition which one fails to duplicate even by the injection of such potent agents as curare or botulinus toxin.

Table V

Winter sickness after prolonged exposure to 6° C.
(*Platysamia cecropia*)

Weeks at 6° C.	No. pupae	Stage of adult development after chilling	Behavior at 25° C.
42	40	0–1 day	Lively; prompt development
44	20	0–1	Lively; prompt development
46	25	0–1	Induced motion only; prompt recovery and development
49	40	0–1	30% show flaccid paralysis and electrical inexcitability at outset; all recovered and developed
52	50	0–1	50% flaccid and inexcitable at outset; all recovered and developed
57	90	0–1	70% flaccid and inexcitable at outset; all recovered and developed into feeble moths
66	40	0–2	All flaccid and inexcitable at outset; only 60% recovered and developed into feeble moths
70	65	0–2	All flaccid and inexcitable at outset; only 50% recovered and developed into feeble moths
74	10	0–2	All flaccid and inexcitable at outset; only 25% recovered and developed into feeble moths
90	50	0–2	All flaccid and inexcitable; none recovered

It was first thought that winter sickness might be peculiar to animals showing early adult development. This prospect was negated by the finding that at 2½° C., in the absence of any trace of adult development, the onset of winter sickness is even more prompt than at 6° C. This same effect was noted in other species. Indeed, in *Samia walkeri* (*cynthia*) winter sickness becomes irreversible after only 7 months at 2½° C.

A clue as to the physiological basis of winter sickness was provided by the following experiment performed on *Samia walkeri* (*cynthia*). From seven individuals showing reversible winter sickness the blood was drained and exchanged with that of a corresponding number of normal diapausing pupae. All animals were then maintained at 25° C. Within 12 hours the normal pupae receiving the winter-sick blood showed full and complete paralysis of the abdominal muscles. Electrical excitability also disappeared and reappeared only 3–5 days later. Recovery was synchronized with the formation of a chalky precipitate, which settled on the plastic window at the tip of the abdomen and also deposited itself in the lumen of wings, antennae, and the body cavity as a whole.

The winter-sick pupae which received normal blood likewise recovered in 3–5 days, though whether this was hastened by the transfusion of normal blood cannot be stated in the absence of suitable controls. No precipitate formed in this blood and none was detected in the adult moths that ultimately formed.

In effect, therefore, it was possible passively to transfer the winter sickness by the transfusion of winter-sick blood. Moreover, the detoxification of this blood was accompanied by the formation of a copious white precipitate, presumably containing ureates and other insoluble end-products of nitrogenous metabolism.

Samia walkeri (*cynthia*) is a species which never escapes from diapause if maintained at temperatures higher than 20° C. After 15 months at 25° C., the normal, lively pupa shows the formation and deposit of the same sort of precipitate as mentioned above. Evidently, the intermediate metabolism of the pupa at the warm temperature is able to cope with nitrogenous end-products by detoxification and "excretion by precipitation," whereas, at temperatures below 10° C., the production of soluble end-products outbalances their detoxification. Winter sickness, in this sense, appears to be a genuine case of auto-intoxication within the "closed system" of the pupal insect, and to bear a resemblance to the mammalian syndrome of uremia. The phenomenon obviously merits further study.

Discussion

On the basis of the experimental results set forth in the preceding sections, it is clear that the catalytic effects of low temperature on the diapausing insect are mediated by an endocrine mechanism. This mechanism centers in the brain. Previous studies had already demonstrated that the brain loses its endocrine powers just prior to the pupal moult and is inactive in the freshly pupated insect (Williams, 1946, 1952). We now see that the rate with which the brain recovers its activity is largely determined by environmental temperature.

By an apparently direct action on the brain itself, temperatures within the range 6 to 15° C. greatly accelerate the functional recovery of the brain. As soon as the brain has undergone this physiological change, the catalytic effects of low temperature are at an end. The pupa, equipped with an endocrinologically competent brain, becomes, in effect, the equivalent of a non-diapausing pupa.

It is fruitful to consider the similarities between two such animals; *i.e.,* between chilled diapausing pupae and unchilled non-diapausing pupae. Thus, in both types of animals the actual secretion of the brain hormone, as well as the development that follows, are favored by high temperature and retarded by low temperatures. Moreover, in both types of animals one can ordinarily establish or re-establish a pupal diapause by prompt excision of the pupal brain (Williams, 1952). And, finally, in both types of pupae, low temperature is still capable of enforcing a developmental standstill which is reversible at higher temperatures. On the basis of this comparison it seems necessary to conclude that the end-point of the pupal diapause is physiological in character, and is signalled by the functional recovery of the brain.

If attention is now directed to the overwintering of the diapausing insect, it is clear that exposure to temperatures within the optimal range of 6 to 15° C. ordinarily continues beyond the 10 weeks required for the full and complete recovery of the brain. Hence, under normal circumstances, the dormant state begins as a "diapause" and continues thereafter as a "quiescence" (Andrewartha, 1952; Lees, 1955). The net effect is that the overwintering insect is poised to react to the warm temperatures of spring.

From the physiological point of view, attention focusses on the brain as the vehicle of the low temperature effect. Indeed, within the substance of the pupal brain, it is reasonable to pin-point the temperature effect even more closely within the 26 neurosecretory cells which secrete the brain hormone. Low temperature apparently accelerates the functional recovery of these cells from their inactive state in the diapausing pupa.

The activating reaction, itself, pursues a typical time-course as dictated by temperature. Critical exposure to low temperature is first required, following which the activation proceeds to completion within a period of several weeks. The unknown reaction within the neurosecretory cells therefore shows the kinetics of a threshold reaction but not of an "all or none" reaction.

We have observed that an intermediate range of low temperatures (6 to 15°C.) is optimal for the physiological transformation of the brain. At temperatures as high as 25° C. or as low as 2½° C. the process is greatly retarded. The fact that 2½° C. is far less effective than 6° C. gives assurance that no single temperature-inhibited reaction (such, for example, as adsorption) will suffice. The minimal requirements appear to be the interaction of two antagonistic reactions with different temperature coefficients, the one favoring and the other opposing the activation of the neurosecretory cells.

Until very recently, the analysis could not be pursued beyond this point. Most fortunately, however, the biochemical and physiological events within the pupal brain of the Cecropia silkworm have just been described in detail by Van der Kloot (1955). In brief, the unchilled diapausing brain is found to be not only endocrinologically incompetent, but also electrically silent—a state which can be accounted for in terms of the apparent absence of cholinesterase from the brain as a whole. The cholinergic substrate of this enzyme (presumed to be acetyl choline) is, at the outset, present in only trace amounts. Exposure to low temperatures greatly accelerates the accumulation of this substrate within the brain. Then, when the chilled pupa is returned to 25° C., the brain shows a prompt, and, presumably, inductive synthesis of cholinesterase. The titer of acetyl choline decreases to an intermediate level, and electrical and endocrine activities immediately reappear. These various changes are peculiar to the brain and are not encountered in any of the other ganglia.

The implications of these extraordinary findings are discussed in detail by Van der Kloot. For present purposes it is of particular interest that the entire brain is shut down at the outset of diapause and reactivated months later under the influence of environmental temperature. What this means in terms of the function of the neurosecretory cells is not clear. Van der Kloot suggests that the entire brain is inactivated to prevent the neurosecretory cells from being driven by nerve impulses at synapses with afferent and internuncial neurons. And one may likewise conjecture that within the neurosecretory cells the synthesis and secretion of hormone continues to require cholinergic mechanisms of the same type as preside over the genesis of nerve impulses and the release of neurohumors.

The primary change induced by chilling therefore appears to be the synthesis and accumulation within the brain of a cholinergic substrate which appears to be acetyl choline. It will be recalled that molecules such as acetyl choline are notoriously unstable in solution at physiological pH, and one may predict that the accumulation of substrate is opposed by non-enzymatic breakdown. Consequently, it is even possible that the synthesis and breakdown of the cholinergic substrate correspond to the pair of antagonistic, temperature-sensitive reactions whose existence was found necessary to account for the endocrinological activation of the brain.

SUMMARY

1. The termination of the pupal diapause of the Cecropia silkworm is potentiated by preliminary exposure to low temperature and, especially, to temperatures in the range 6 to 15° C.

2. This effect is mediated by an endocrine mechanism which centers in the brain, and is specifically concerned with restoring the competency of the brain's neurosecretory cells to synthesize and secrete the brain hormone.

3. The time required for the initiation of adult development at 25° C. is thereby reduced from 27 weeks to as little as one day.

4. As soon as the brain has recovered its endocrine function, the catalytic effects of low temperature are at an end; the actual secretion of the hormone and the developmental response that follows are then favored by high temperature. In this manner the low temperatures of winter potentiate the initiation of adult development the following spring.

5. The effects of low temperature on the inactive, diapausing brain are considered in detail. The action is apparently a direct one on temperature-sensitive reactions within the brain itself. The over-all process of activation shows the kinetics of a threshold reaction but not of an all-or-none reaction.

6. These findings are discussed in the light of Van der Kloot's chemical and physiological studies of the brain of Cecropia. Within the substance of the brain the primary change induced by low temperature appears to be the synthesis and accumulation of a cholinergic substrate.

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A BIMODAL RESPONSE TO DIETARY SUGARS BY AN INSECT[1]

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The gustatory responses of different animal species to sugars have been studied by many workers, and the apparent universality of animal acceptance of sucrose has been pointed out by Frings (1946). The biological importance of carbohydrates and their lack of olfactory-stimulating properties makes them well suited for experimental studies of gustatory responses. As discussed by Roeder (1952), insects are excellent experimental animals for studies in several areas of sensory physiology, owing to their small size, ease of culturing in large numbers, and relative simplicity of behavior. Several different aspects of the gustatory reactions of insects to sugars have been investigated: taste-acceptance thresholds have been determined; the influence of sugars on feeding behavior has been studied; and the relationships between nutritive value and taste-acceptability have been investigated (for review see Dethier, 1953).

As part of a study of the behavior of the larva of the European corn borer, *Pyrausta nubilalis* (Hübn.), on its principal host plant, an investigation was undertaken of the influence of dietary sugars on larval feeding behavior under controlled experimental conditions. The larvae were found to be very sensitive to sugars (Beck, 1956a), and the concentration of sugars in the tissues of the corn plant was shown to influence the orientation of the insect on the plant (Beck, 1956b). The experimental method developed during the course of this research made possible the quantitative measurement of the influence of gustatory substances on feeding behavior. The nature of the measured feeding response and a discovered bimodality of the insect's response to low concentrations of sugar constitute the subject matter of the present report.

METHODS

The experimental method employed was of a multiple choice design, in which newly hatched, unfed European corn borer larvae were allowed to choose the dietary media on which to feed. The agar-based medium shown in Table I was used as the nutritional substrate. Agar-gel alone was of a consistency unacceptable to the insects; the addition of casein and cellulose resulted in a medium on which the larvae would feed. The amounts of sugars employed in these experiments were far too minute to necessitate the adjustment of the amounts of the other constituents to compensate for the slight changes in volume and concentration.

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The media were mixed thoroughly with a motor-driven stirrer while being held in a boiling water bath, and then poured into Petri plates (15 mm. × 90 mm.) to gel. From the gelled media, round discs were cut by means of glass tube 18 mm. in diameter. The discs were transferred to Petri plates where they were placed at equal distances apart in a circle, the center of which was the center of the dish. The discs of diet so placed are referred to hereafter as feeding stations. In two-choice experiments there were four feeding stations per Petri dish, each medium being represented twice. The similar media were placed opposite each other in the four-membered circle. In four-choice experiments, eight feeding stations were present, each medium being represented at two stations, and the relative positions of the different media were randomized according to a table of random numbers. The position-randomization of the media in four-choice experiments was modified only to the extent that two identical diets were never placed adjacent to each other. This modification of the random distribution was found to be necessary in order to avoid position effect errors. About 40 European corn borer eggs in the black-head stage (and about four hours from hatching) were placed on a small piece of moist blotting paper at the center of dish. The lids of the Petri dishes were lined with tightly fitting discs of blotting paper to aid in preventing the larvae from escaping. Eight replicate Petri dishes were set up in each experiment. After the experiment was set up, the dishes were placed in the dark at 30° C. for 24 hours. At the end of this time, the dishes were examined and the number of larvae established at each feeding station was recorded. No counts were made of larvae not on feeding stations. The counts of the number of larvae established at each feeding station were interpreted as reflecting the influence of the experimental diets on the maintenance of larval feeding over the experimental period. Preliminary experimentation demonstrated that the distribution of larvae among the feeding stations was random when the feeding stations were of identical diets.

TABLE I

Composition of agar-based media used to test the feeding reactions of newly-hatched larvae of the European corn borer

Substance	Amount (g)
Bacto-agar	1.10
Fibrous cellulose	0.50
Powdered cellulose	2.00
Casein, vitamin free	3.00
Glucose (or other sugar)	variable
Distilled water	40.00 (ml.)

The physical characteristics of the various media were found to be quite uniform. The larvae were found to be relatively insensitive to small differences in pH and water content; small variations in these properties did not represent a source of experimental error. It was found unnecessary to maintain aseptic conditions in the Petri dishes when the incubation period was 24 hours. With a 48-hour incubation period, the use of aseptic techniques was necessary. However, the larval establishment data at 24 hours were indistinguishable from those at 48 hours, so the shorter time and simpler technique were adopted.

The experimental data were treated statistically by analysis of variance, after being subjected to square root transformation as recommended by Snedecor (1946) for treatment of small numbers. Regressions were calculated by the method of least squares.

Results and Discussion

The nature of response to sugar. Four-choice experiments, in which the larvae were offered dietary media containing different amounts of glucose, yielded re-

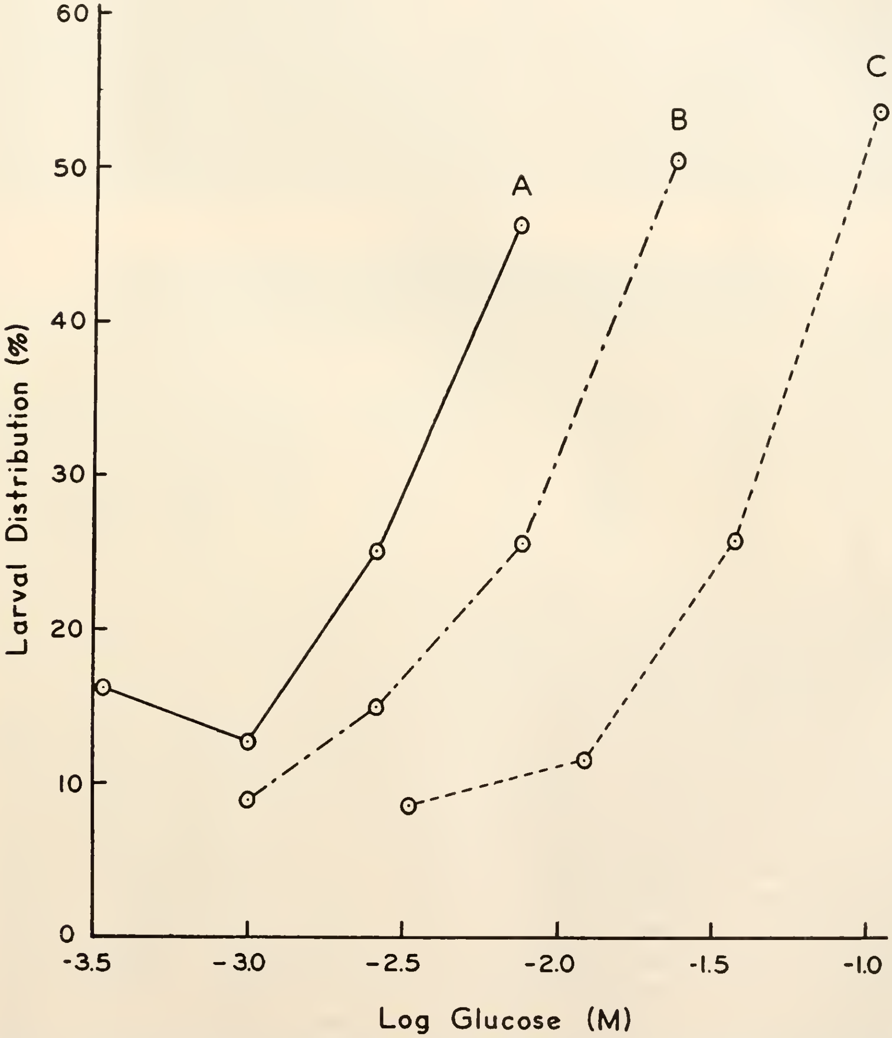

Figure 1. Three experiments (A, B, and C) on the effect of glucose concentration on the distribution of European corn borer larvae among dietary media (four-choice experiments).

sponse curves such as those shown in Figure 1. Over the range of glucose concentrations tested, the larvae tended to concentrate on the highest sugar level available to them. Similar response curves were obtained with sucrose and fructose. The percentage of the larvae established on a given feeding station was apparently independent of the amount of glucose actually present in the medium, but was dependent upon the concentration relative to the concentrations offered in the other media present.

Early in the investigation it became obvious that interpretation of response data would be dependent upon an understanding of the mechanisms by which the larval population became distributed among the feeding stations in proportion to the relative sugar content of the media. Observations on the behavior of newly hatched borer larvae showed that they were initially attracted to the agar-based media, even when the media were devoid of sugars or other nutrients. Such behavior was probably the result of a hydrotactic response. It was also observed that the larvae were quite active up to at least 24 hours after hatching and tended to wander from feeding station to feeding station. They displayed much greater tendency to wander away from stations low in sugar than from those containing high sugar levels, however. Because of the larvae's sensitivity to light, it was not possible to determine by direct observation the exact influence of sugar concentration on the duration of larval feeding.

TABLE II

The effect of time of observation on determinations of the influence of glucose on the establishment of newly hatched European corn borer larvae

Time after eclosion (hr.)	Larval establishment		Statistical significance of difference
	control—no glucose (%)	glucose 0.004 M (%)	
2	49.3	50.7	not significant
6	29.1	70.9	highly significant
12	28.6	71.4	highly significant

On the basis of the observations on larval behavior and the relative nature of the experimental data, the hypothesis that the length of time that a larva remains at a given feeding station is in proportion to the sugar concentration in that station was tested experimentally. Experiments were run in which the effect of the presence or absence of glucose on the distribution of the larvae between two media was measured at different times after the eggs hatched. The results of such two-choice experiments are shown in Table II. The data show that the initial larval distribution between the media was random. By six hours after hatching, the random distribution had been replaced by a non-random distribution in which most of the larvae were found on the feeding stations containing glucose. Although the percentage distribution remained materially constant after six hours, the larvae tended to wander intermittently up to well over 24 hours. At about six hours after hatching, the larval distribution had reached an equilibrium, in which the number of larvae vacating a given feeding station per unit time was equal to the number arriving from other stations. If the ratios of larval distribution among the media are directly related to the ratios of the average times the larvae remained on the different media, it should be possible to reproduce the experimental data by calculating the equilibrium in time. Table III shows such a calculated distribution equilibrium. Very good agreement between observed and calculated data was obtained. It is apparent that response data, such as shown in Figure 1, represent larval distributions at equilibrium. The percentage distribution of the larvae at the point of equilibrium must be relative to the single experimental variable of sugar concentration, and the distribution data are in agreement with the hypothesis that the response data measure the ratios of the average larval feeding times on the different sugar concentrations.

The general relationship between dietary sugar (glucose) concentration and duration of larval feeding was estimated by the assumption of arbitrary time units in which the average larval feeding time on a just subliminal concentration (0.001 M) of glucose was taken as one feeding time unit. Feeding time values were then calculated from larval distribution ratios obtained in a series of two- and four-choice experiments. The regression of feeding time on glucose concentration obtained by this method is shown in Figure 2. The value of the feeding time unit, as here defined has not been accurately determined, and the best approximation now possible is that it lies between 20 and 45 minutes. Its value in conventional time units is of little importance, as it will, undoubtedly, change with varying experimental conditions.

TABLE III

Comparison of larval distribution observed in a four-choice experiment with calculated distribution equilibrium

Feeding stations:	A	B	C	D
Larval distribution *observed* (%):	13.0	17.5	27.5	42.0
Distribution ratio (DR) *observed*:	1.00	1.38	2.15	3.38
Larvae leaving station per unit time (1/DR×100) *calculated* (%):	100	72.5	46.5	29.6
Larval distribution at equilibrium *calculated* (%):	12.7	17.5	27.6	42.2
Larval distribution difference (*observed—calculated*):	0.3	0.0	−0.1	−0.2

The importance of feeding time in the response of insects to sugars is indicated by the published results of several other workers. Kunze (1927) found that the duration of feeding by honey bees could be used as an index of the acceptability of sugar solutions to this insect. Weis (1930) reported that the length of time of uninterrupted feeding was proportional to the sugar (sucrose) concentration of the liquid fed upon by the butterfly *Pyrameis atalanta* L. The volume of liquid ingested per feeding by the honey bee was observed to be directly proportional to the dietary concentration of sucrose (Von Frisch, 1935). Dethier and Rhoades (1954) described certain relationships between the volumes of liquid ingested by adult blowflies and the sugar content of the liquids employed. Using adult blowflies, Frings and Frings (1946) demonstrated that the volume ingested is directly proportional to the duration of feeding. The tendency for an organism to concentrate its feeding on nutritional substrates high in sugar content has been termed *saccharotrophism* (Beck, 1956b).

From the results presented above, it is apparent that saccharotrophism in the European corn borer, and probably in some other insects, is the influence of sugar concentration on duration of feeding. The experimental data constitute quantitative measurements of the intensity of the saccharotrophic response. The data are not a measure of the proportion of the insect population responding to the sugar concentrations tested. Weis (1930) questioned the reliability of using feeding duration as a quantitative measure of acceptability because of the uncontrolled variables of age, water balance, and nutritional history of the experimental animals. In the present study these factors were standardized by the use of newly-hatched

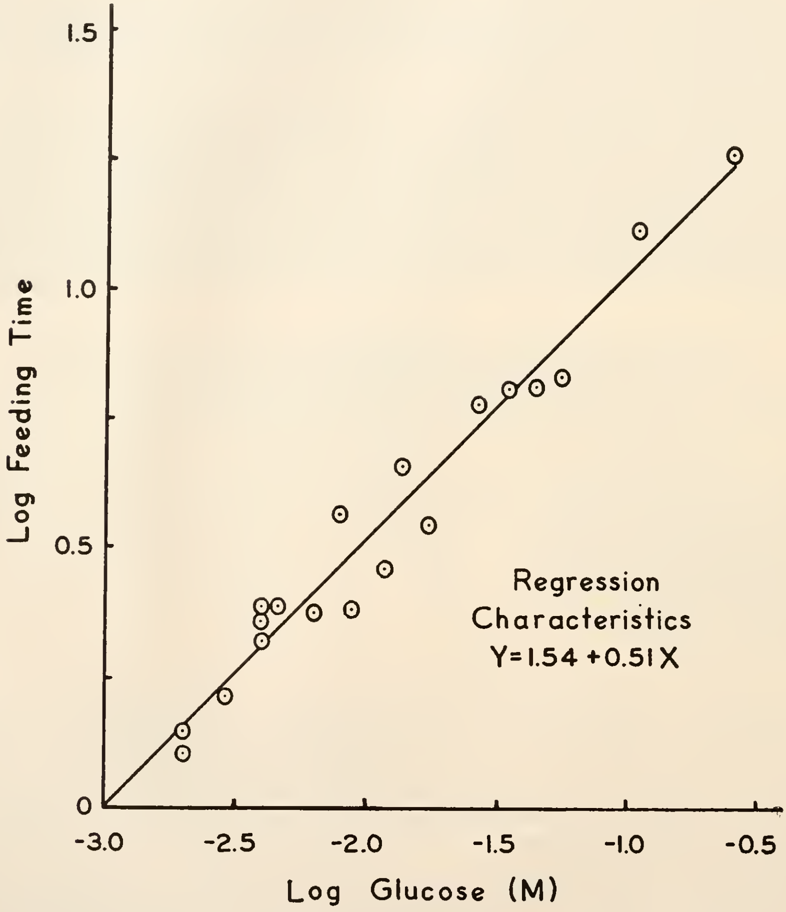

FIGURE 2. The effect of glucose concentration on the duration of feeding by newly hatched larvae of the European corn borer.

and previously unfed larvae, and under these conditions the European corn borer larvae employed represented a nearly ideally uniform experimental population.

Bimodal response. During the course of running a series of four-choice feeding experiments designed to allow an estimation of the feeding acceptance thresholds for glucose, sucrose, and fructose, response curves similar to curve A of Figure 1 were frequently obtained. Such curves, in which the response to the lowest sugar concentration was higher than to the next higher concentration, were at first considered to be the result of the statistical vagaries of the experimental method. As more data were obtained, it became evident that responses of this nature were consistently observed in any experiment which included two sugar concentrations somewhat below the apparent acceptance threshold. This phenomenon was investigated in greater detail by means of a series of two-choice experiments in which one of the pair of diets used was devoid of sugars and the other contained a known concentration of glucose. The results are shown in Figure 3. Each point shown in this figure is the mean of the results of from three to five experiments, each of which was made up of eight replicates. Statistical analyses of the data showed that the response to the diets containing sugar was significantly different from the response to the control diets at all sugar concentrations except 0.0001 *M* ($\log = -4.0$) and 0.001 *M* ($\log = -3.0$). At concentrations below 0.001 *M* but above 0.0001 *M*, the larvae tended to concentrate their feeding on the control diets (no sugar) in preference to the diets containing such low levels of sugar. Although the response differences were small, they were reproducible and statistically significant. These data indicate a bimodal response to glucose concentration. At concentrations above an apparent threshold of approximately 0.001 *M*, the feeding response (time) was directly proportional to the sugar concentration and the data are consistent with the Weber-Fechner law. The larval reaction toward concentrations lower than 0.001 *M* was opposite in sign, in that it reduced the feeding period below that elicited by diets containing no sugar. The threshold for the negative response appeared to lie between 0.0001 and 0.0002 *M*. The negative response toward concentrations below the threshold for a positive feeding response was observed in experiments with fructose and sucrose, as well as with glucose. It is therefore apparent that it is not a reaction associated with glucose only. Different manufacturer's lot numbers of glucose (A.C.S. specifications) gave identical results, so the reaction was not a response to the purity of the sugar preparations.

The physiological mechanisms underlying the observed bimodality have not been determined. The data do not necessarily imply a bimodality of response of sugar-sensitive receptor organs, as the present investigation has dealt with only behavioral aspects of the problem. The data show that: (1) the response of European corn borer larvae to different concentrations of sugars is measurable and can be expressed quantitatively in terms of the effect of sugar concentration on the duration of larval feeding. (2) As the sugar concentration is increased above a threshold value lying between 0.0001 *M* and 0.0002 *M*, there is a proportionate increase in the duration of feeding. (3) The response data show a bimodal characteristic in that larval feeding on diets containing low suprathreshold concentrations of sugar is of shorter duration than on diets containing subthreshold amounts of sugar. The following interpretation of these findings is offered, although its speculative nature must be recognized. The duration of feeding is probably related to the time required for the insect to become adapted to the diet. When in contact with a diet containing no sugar (or a subliminal concentration), stimulation of sugar receptors does not occur, and these sense organs can play no role in determining feeding duration. When they are stimulated, however (as by a suprathreshold sugar concentration), feeding maintenance is dominated by the impulses they feed into the nervous system. Such impulses cease as the larva becomes

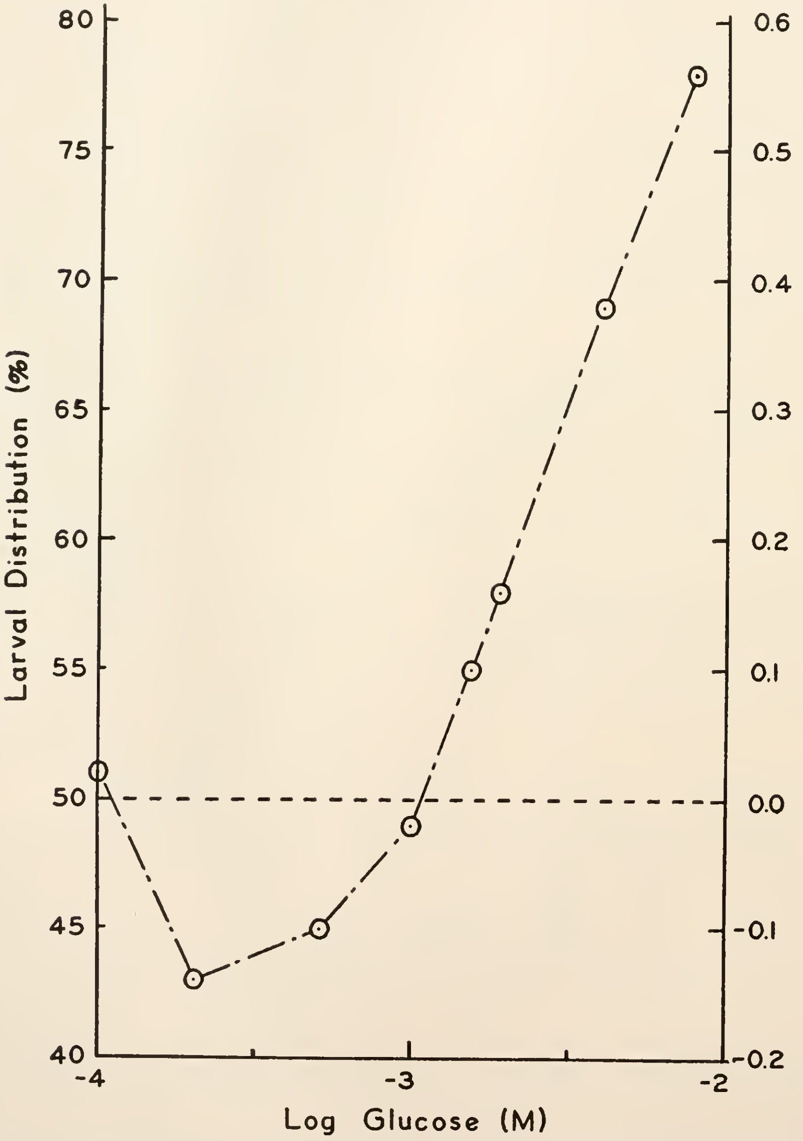

FIGURE 3. Feeding response spectrum of European corn borer larvae for glucose. Two-choice experiments. Plotted data do not include responses to control (no sugar) media.

adapted to the diet, and the behavior pattern undergoes a shift. The lower the sugar level, the more rapid the adaptation, and the shorter the feeding period. In the absence of supra-threshold sugar concentrations, duration of feeding must be determined by other factors. A bimodal response would be obtained experimentally under conditions where duration of feeding is more sensitive to sugar than to alternative factors influencing behavior in the absence of sugars.

A bimodal response to low sucrose concentrations has been reported in human tests by Neuman (1944). Working with human subjects, she found that the quality of sensation caused by sucrose was dependent upon intensity of the stimulus. Her subjects reported that the perceived sensations from sucrose solutions near the threshold level of concentration may be a complex of sweet, bitter, and/or sour. Samuel Renshaw (personal communication) determined human taste spectra for sucrose and found that at the lowest threshold, sucrose gives rise to a smooth tactual sensation rather than to a sweet taste. As the concentration is progressively increased, the taste is bitter, then bitter-sweet, and finally purely sweet. Although it is not possible to determine the quality of sensation produced in an experimental animal, the response data obtained by use of European corn borer larvae show a response spectrum to sugars that is strikingly parallel to the human taste spectrum for sucrose.

The writer is pleased to acknowledge his indebtedness to Mr. E. T. Kaske for technical assistance in conducting the experiments, and to Professors V. G. Dethier and S. Renshaw for their cooperation and review of the manuscript.

SUMMARY

1. A study of the feeding behavior of newly hatched, previously unfed larvae of the European corn borer has shown that dietary sugar concentration has a pronounced and quantitatively measurable effect on such behavior.

2. The larvae tended to prolong their feeding in proportion to the dietary sugar concentrations.

3. The responses to sugar concentrations above a positive response threshold of about 0.001 M were in agreement with Fechner's law over the concentration range tested. There was a small but significant negative feeding response toward sugar concentrations below 0.001 M and down to 0.0002 M.

4. The experimental results obtained indicate that this organism displays a bimodal response to glucose, sucrose, and fructose, in which the modes differ in sign and are dependent upon the concentrations employed. The response spectrum of the insect toward the sugars tested appears to be parallel, if not directly comparable, to the human taste spectrum for sucrose.

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A HISTOLOGICAL AND CYTOPHOTOMETRIC STUDY OF THE EFFECTS OF X-RAYS ON THE MOUSE TESTIS[1]

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Studies of Bergonié and Tribondeau (1904), Regaud and Blanc (1906) and others focussed attention on irradiation-induced changes in reproductive organs and gametes of various organisms. Their findings indicate that spermatogonia are the most x-ray-sensitive components of the seminiferous epithelium and that, following the decrease in numbers of spermatogonia, the other cell types disappear in the order of their development. The topic has been the further subject of numerous publications and the literature has been reviewed by Heller (1948).

Investigations of the nature of x-ray-induced sterility in the male mouse have been carried out by Snell (1933) and by Hertwig (1938). Recent reviews by Kaufmann (1954) and Russell (1954) have dealt with radiation-induced changes of cytological and genetic interest.

Recently, emphasis has been given to quantitative histological studies of irradiated testes. Studies have been carried out by Eschenbrenner, Miller and Lorenz (1948), Eschenbrenner and Miller (1950), Fogg and Cowing (1951), Barrow and Tullis (1952), Shaver (1953) and Oakberg (1955). Investigations have been facilitated by the histological scoring procedure developed by Chalkley (1943) and by the cytological studies of Roosen-Runge and Giesel (1950), and of Leblond and Clermont (1952).

The present approach differs from the above in that it represents the combination of a quantitative histological method together with cytophotometry, thus affording data with respect to the DNA content of irradiated tissues as well as changes in the frequency of cells.

MATERIALS AND METHODS

Two inbred strains of mice, differing in their sensitivity to mouse typhoid, were used in these experiments. The animals chosen were 58-day old males of strains BALB/Gw (hereinafter designated as Ba) and S. The animals were irradiated in plastic tubes and were exposed to a dose of 320 r (250 pkv, 30 ma; filtration 0.25 mm. Cu, 1 mm. Al; anode-target distance 47.5 cm., dose rate 430 r/min.). The irradiation was delivered to the pelvic region only, the rest of the body being shielded with lead.

Control and irradiated animals were killed at 1, 8 and 24 hours, 3, 5, 10, 16 and 28 days after irradiation. From each animal one testis was removed, quickly freed of associated tissues, rolled on filter paper, weighed on a Roller Smith torsion bal-

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ance and dropped into fixative. The other testis was removed and treated in a similar manner, and was later used for dry weight determinations. The testis in fixative was then cut into several pieces. Very few minutes elapsed from the time each animal was killed until the testis removed into fixative was subdivided.

In the case of the one-hour controls, kidney tissue also was fixed and later served as a control tissue for the Feulgen reaction.

Fixation

All tissues were fixed in Carnoy's acetic-alcohol (1:3) for three hours, washed in several changes of absolute alcohol, cleared in benzene and embedded in 56–58° C. Tissuemat. Sections were cut at 7 and at 10 μ. For photometric purposes, sections from all animals of one strain were mounted on the same slide together with sections of kidney obtained from a Ba control animal.

Staining

The desoxyribose nucleic acid (DNA) content of nuclei was visualized by means of the Feulgen reaction. The reagent was prepared according to the method of Stowell (1945) and sections were stained for two hours at room temperature following hydrolysis (12 minutes) in 1 N HCl at 60° C. Some Feulgen preparations were lightly counterstained with fast green and used for cytological studies. Unhydrolyzed slides were immersed in the Feulgen reagent and served as controls for photometric purposes.

Other slides were stained with a mixture of safranin and fast green as described by Bryan (1955). These preparations were used for routine cytological and histological studies as well as for the quantitative scoring procedure described below.

Additional slides were stained by means of the periodic acid-Schiff (PAS) reaction. The reagents were prepared according to McManus (1948) and the slides were stained as described by Bryan (1954), methyl green being used as a counterstain. This procedure was used, in the earlier phases of this work, as an aid in the identification of the developmental stages of the seminiferous epithelium, after the manner described by Leblond and Clermont (1952). It should be mentioned, however, that the stages of development of the seminiferous epithelium as described for the rat do not entirely correspond to the stages observed in the mouse.

Photometry

Measurements of the DNA-Feulgen complex were made with an apparatus essentially the same as that described by Pollister (1952) and others. A tungsten filament lamp (6 volt, 18 amp.) was used as a light source and the spectral lines isolated by means of a Perkin-Elmer monochromator equipped with appropriate accessory optics.

It was necessary to compare data obtained from several slides. Therefore, sections of control kidney were used as a standard to check on the reproducibility of the Feulgen reagent. Means of the DNA-Feulgen content of kidney nuclei obtained from the different slides did not differ significantly from each other.

Transmittance data were obtained by the "plug" method described by Swift

TABLE I[2]

Frequency of cell types at various times after 320 r of x-rays

Stage and strain		1 hour	8 hours	1 day	3 days	5 days	10 days	16 days	28 days
Spermatogonia in interphase	Ba	102.6	110.3	91.0	1.1	9.2	10.4	50.1	161.3
	S	107.8	101.5	79.2	2.2	0.0	3.3	37.7	187.6
Spermatogonia in mitosis	Ba	82.9	26.8	65.9	4.9	12.2	9.8	56.1	173.2
	S	102.6	11.5	21.5	0.0	0.0	2.3	26.4	222.8
Spermatocyte chromatin	Ba	96.3	98.3	109.3	116.7	92.4	64.8	10.0	110.5
	S	96.3	101.6	107.6	112.1	97.2	82.8	0.0	122.2
Spermatid nuclei	Ba	95.1	97.0	87.3	93.4	122.5	144.4	126.4	49.8
	S	111.8	95.5	99.7	106.0	132.5	144.1	149.5	23.1
Sperm heads	Ba	112.8	105.2	113.8	134.7	146.6	228.4	369.7	51.6
	S	89.6	107.6	97.0	112.2	132.4	246.7	358.3	63.4
Sertoli nuclei	Ba	104.9	110.0	97.0	72.8	96.5	125.4	139.1	115.1
	S	99.3	85.9	104.7	113.4	125.5	160.4	125.9	136.6

[2] Values expressed as per cent of controls.

(1950), and were converted into extinction values ($\log_{10} {}^1/T$). From the extinction data, the amounts of the DNA-Feulgen complex were computed as described by Swift (1950). In most cases the nuclei selected for measurement purposes were located beforehand and their location indicated on a camera lucida map.

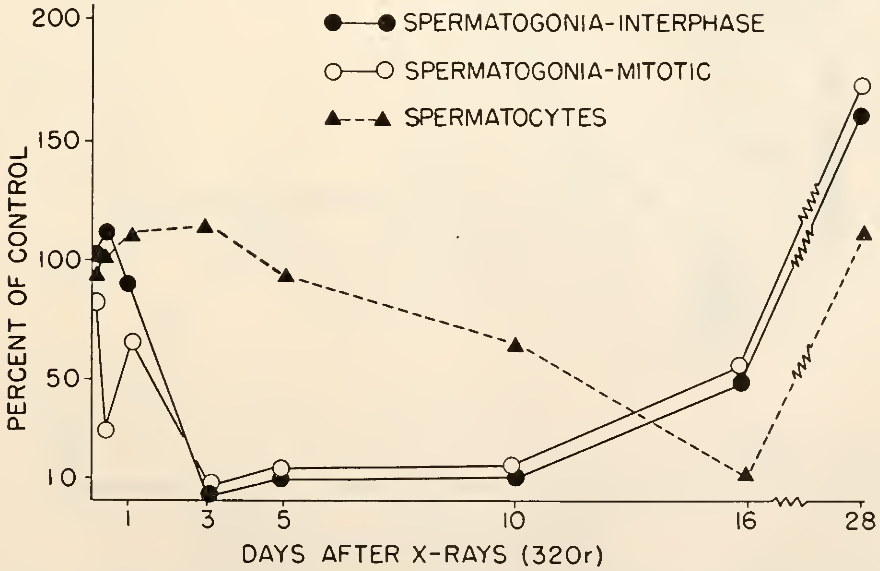

FIGURE 1. Strain Ba. Incidence of spermatogonia and spermatocytes at different times following 320 r of x-rays.

Scoring of cells

Estimates of the relative areas of the seminiferous tubules occupied by each stage of spermatogenesis were obtained by means of the quantitative scoring procedure developed by Chalkley (1943). The manner in which this procedure was employed was based on a similar application by Eschenbrenner, Miller and Lorenz (1948) but with minor modifications.

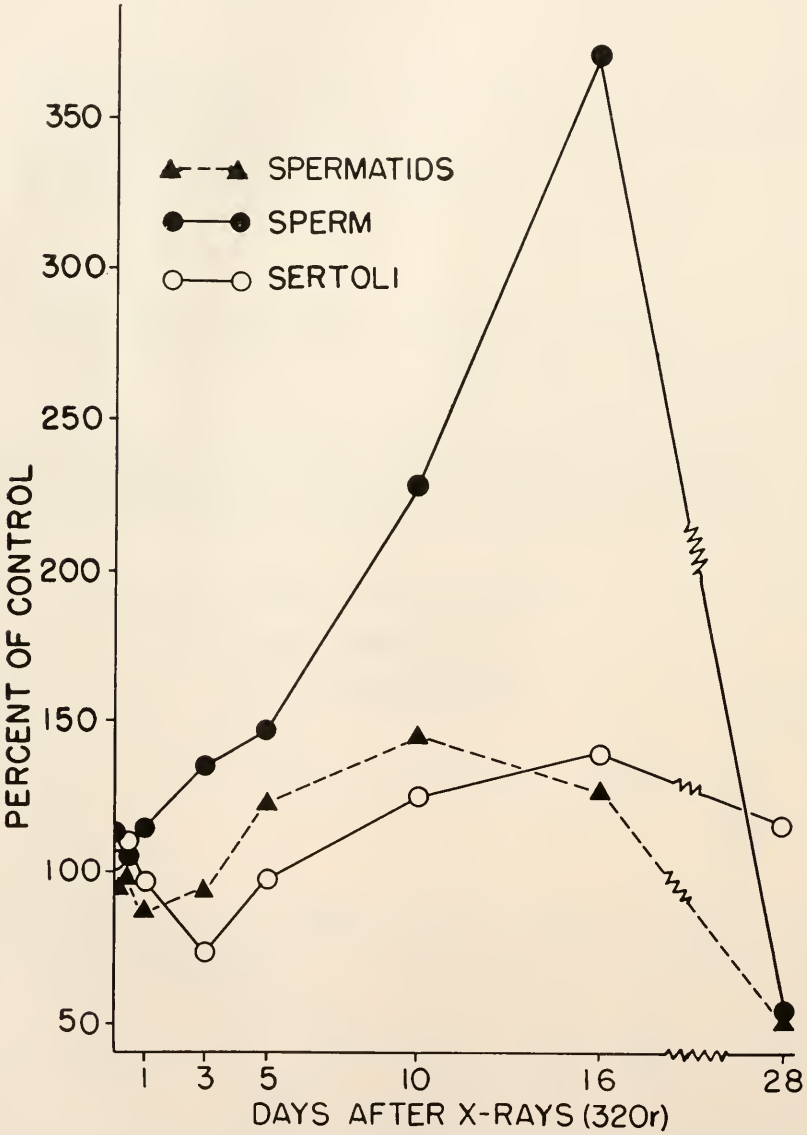

FIGURE 2. Strain Ba. Incidence of spermatids, sperm and Sertoli nuclei at different times following 320 r of x-rays.

Nuclei were recorded as belonging to the following classes: interphasic spermatogonia, dividing spermatogonia, spermatocytes, spermatids, sperm and Sertoli nuclei. Eschenbrenner *et al.* did not record "hits" on Sertoli nuclei and recorded only spermatids possessing spherical nuclei and only those sperm heads evincing "well defined dorsal fins." In the present work, all stages of spermatid development and of sperm maturations were recorded. On account of these differences in procedure, the results of Eschenbrenner *et al.* and those reported here are not strictly comparable.

RESULTS

A. Histological studies

The histological scoring data are summarized in Table I and are presented in graph form in Figures 1–4. In Table I the data pertaining to both strains are presented together in order to facilitate their comparison. All values in this table are expressed in terms of per cent of control values.

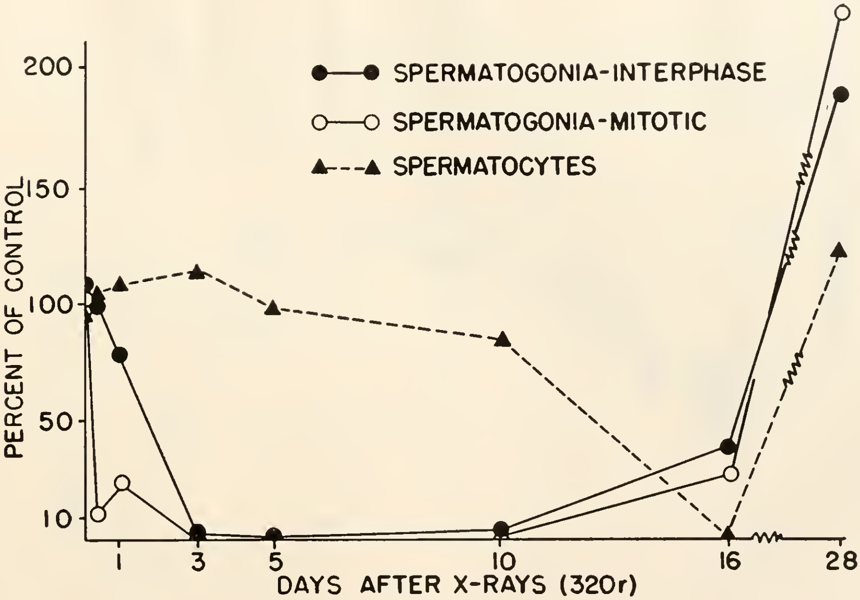

FIGURE 3. Strain S. Incidence of spermatogonia and spermatocytes at different times following 320 r of x-rays.

These data indicate that with the exception of the dividing spermatogonia, there is no pronounced change in the cellular composition of the seminiferous epithelium during the first 24 hours following irradiation. During the period of 1–3 days after irradiation there occurs a marked decline in the area occupied by interphasic spermatogonia. Some regeneration of spermatogonia is apparent by 10 days and is well underway by 16 days. Dividing spermatogonia, on the other hand, undergo a rapid decrease during the period of 1–8 hours after irradiation. At 24 hours,

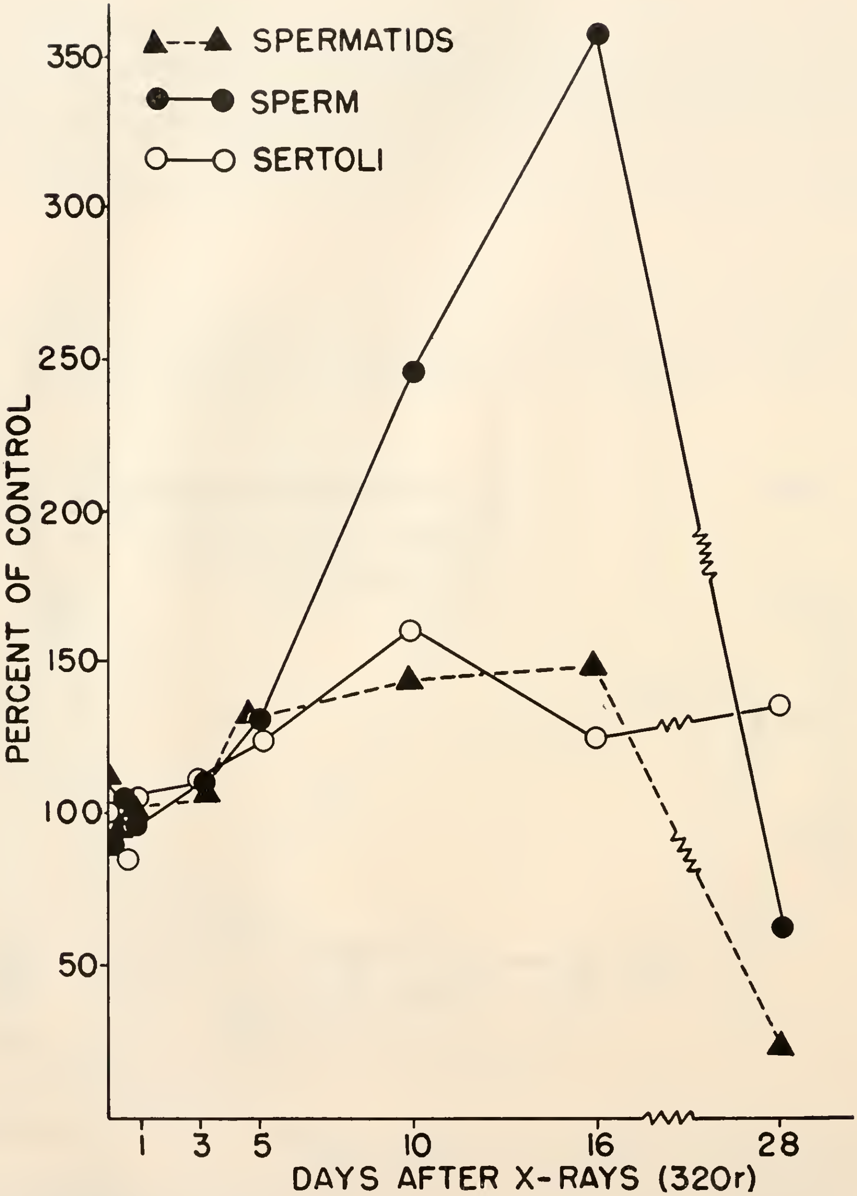

Figure 4. Strain S. Incidence of spermatids, sperm and Sertoli nuclei at different times following 320 r of x-rays.

the area occupied by these cells shows a moderate increase (from 26.8% of the control value at 8 hours to 65.9% of the control value at 24 hours in strain Ba, and from 11.5% to 21.5% in the case of strain S). This increase is only a transient feature since by three days the area occupied by dividing spermatogonia has further declined to a level of less than 5% of the control value. Thereafter, the dividing spermatogonia show essentially the same behavior as the interphasic cells.

During the course of the histological studies it was found that the seminiferous epithelium of both control and irradiated animals contained cells in various stages of degeneration. The frequency of such cells appeared to be greater in the irradiated material taken from the animals killed during the early part of the post-irradiation period. Accordingly, the pertinent sections were analyzed by means of the Chalkley procedure and the data (expressed as percentages) obtained, from both strains, are presented in Table II.

B. DNA-Feulgen

The data obtained are summarized in Tables III and IV. The values represent the mean amounts of the DNA-Feulgen complex (in arbitarary units) together with their respective standard errors. Nuclei possessing the diploid amount of DNA are designated as Class II nuclei, those with twice this amount Class III, and Class IIa represents nuclei possessing an amount of DNA intermediate between these levels.

Table II

Spermatogonial and non-spermatogonial necrosis after 320 r of x-rays

Strain	Time after irradiation	% spermatogonial necrosis	% non-spermatogonial necrosis	% total necrosis[3]
	Control			
Ba		3.75	2.07	2.27
S		4.03	2.52	2.76
	1 hour			
Ba		3.40	2.99	3.05
S		5.76	2.37	2.83
	8 hours			
Ba		9.71	2.24	2.98
S		8.80	1.68	2.56
	24 hours			
Ba		7.41	2.26	2.79
S		4.55	2.21	2.36

[3] $\frac{\text{No. of necrotic cells}}{\text{total cells scored}} \times 100.$

The range of values for the DNA classes was determined as follows. In the case of the control kidney tissue, the highest value obtained was found to be about 50% higher than the lowest (values ranged from 1.78 units to 2.69 units). The Feulgen-stained kidney nuclei have a more uniform appearance than the spermatogonial nuclei and consequently the spread of values in the latter is somewhat larger with amounts ranging from 1.91 units to 2.94 units. As an approximation to the range expected for Class III nuclei these values may be doubled, yielding a range of from 3.82 to 5.88 units. In actual fact the values for these nuclei were found to range from 3.9 units to 5.6 units but with most of the nuclei distributed over a range of 4–5 units. Thus the spread of values is rather lower than expected. It may be accounted for on the basis of distributional error since many of these nuclei (which are slightly larger than Class II nuclei) show clumping of the stained material. Those values falling between the upper limit of Class II and the lower limit of Class III were assigned to Class IIa.

C. *Other observations*

The irradiated material was examined for indications of damage to the chromosomes. No indications of extensive damage, at the cytological level, were seen.

Table III

DNA-Feulgen content of spermatogonia, strain Ba

Tissue and treatment	Class	No. of nuclei	DNA-Feulgen content
Kidney—control	II	54	2.15±0.03
Testis—control	II	96	2.42±0.04
	IIa	71	3.51±0.04
	III	46	4.52±0.06
Testis—320 r 1 day	II	24	2.57±0.05
	IIa	24	3.44±0.05
3 days	(No nuclei suitable for measurement)		
5 days	II	31	2.52±0.05
	IIa	11	3.30±0.09
	III	1	4.32
10 days	II	29	2.52±0.05
	IIa	12	3.36±0.06
	III	7	4.26
16 days	II	52	2.28±0.07
	IIa	24	3.41±0.05
	III	4	4.16
28 days	II	38	2.54±0.05
	IIa	35	3.50±0.05
	III	7	4.30

The frequency of anaphase figures showing one or more bridges appeared to be very low. During the early period of regenerative activity "nests" of spermatogonial cells were occasionally seen containing one or (rarely) more anaphase bridges. The underlying mechanism responsible for this is not understood; it would appear that in some instances the irradiated spermatogonia must have divided at least once before the chromosomal aberrations showed up.

Other instances of cellular (or nuclear) damage were also observed. Thus, giant nuclei were found in some tubules of the irradiated material. These nuclei made their appearance at about 5 days after irradiation and persisted until 16 days, reaching an apparent high point at 10 days. In addition a few of the tubules examined were found to contain cysts composed of 4 to 8 spermatogonial-like nuclei. The fate of these structures is unknown.

At 16 days after irradiation, spermatozoa occupy about 40% of the area of the seminiferous epithelium. It is of interest that some sperm heads present an abnormal appearance. In contrast to the controls, where the Feulgen-positive material (DNA) is distributed in a relatively homogeneous manner, the abnormal sperm heads have a "bubbly" structure. Such sperm may have been derived from irradiation-damaged spermatids or, possibly, spermatocytes.

Table IV

DNA-Feulgen content of spermatogonia, strain S

Tissue and treatment	Class	No. of nuclei	DNA-Feulgen content
Kidney (Ba)—control	II	30	2.11±0.04
Testis—control	II	40	2.46±0.05
	IIa	47	3.41±0.04
	III	13	4.29±0.07
Testis—320 r 1 day	II	37	2.66±0.04
	IIa	46	3.34±0.03
	III	2	4.73
3 days	(No nuclei suitable for measurement)		
5 days	(No nuclei suitable for measurement)		
10 days	II	21	2.36±0.09
	IIa	6	3.57
16 days	II	44	2.48±0.05
	IIa	24	3.37±0.04
	III	2	4.22
28 days	II	53	2.50±0.05
	IIa	25	3.26±0.03
	III	2	4.20

Discussion

In addition to histological and genetic approaches, the effects of irradiation on biological systems have been studied from the biochemical viewpoint with the aid of tracer techniques. Thus Holmes (1947) used P^{32} in his studies of the effect of x-rays on the synthesis of nucleic acids in the rat Jensen sarcoma. He found that irradiation markedly inhibited the uptake of P^{32} into the DNA fraction of the tumor. Studies by Hevesy (1949) have suggested that the delay in mitosis following irradiation is related to the marked inhibition of DNA synthesis, as measured by C^{14} uptake into DNA purines. It has been shown also, by Abrams (1951), using glycine-1-C^{14}, that irradiation greatly reduces the incorporation of radioactive carbon into the DNA and PNA purines of rat and rabbit intestine, whereas the synthesis of proteins is relatively unaffected. Similarly, Smellie *et al.* (1955) also find marked inhibition of P^{32} incorporation into the nucleic acids of normally mitotically active rabbit tissues following irradiation with 1000 r of x-rays.

Recent work by Klein and Forssberg (1954), using the Ehrlich mouse ascites tumor, indicates that 1250 r of x-rays completely suppresses mitotic activity for a period of 16 hours without any increase in the cell death rate. A linear increase of cell volume with time followed the irradiation treatment. By chemical means, these workers showed that PNA and total nitrogen per cell increased at nearly the same rate as cell volume; DNA, however, did not show this behavior. Later studies by Forssberg and Klein (1954), using glycine-2-C^{14}, point in the same direction, that is, that irradiation brings about a marked decline in DNA synthesis, and, simultaneously, inhibits mitotic activity.

With the aid of a cytophotometer it is possible to measure the DNA-Feulgen content of individual spermatogonial nuclei in interphase. The data presented in Tables III and IV show that in the controls three classes of nuclei are present—those possessing the diploid amount of DNA-Feulgen complex (Class II), the tetraploid amount (Class III) and nuclei containing an intermediate amount of DNA (Class IIa). This latter group is representative of those nuclei engaged in the reduplication of their chromatin content in preparation for the next division, whereas Class III represents spermatogonial nuclei in which synthesis is complete, while the changes involved in the initiation of prophase have not yet occurred.

Irradiation brings about an inhibition of DNA synthesis, as measured by the uptake of radioactive precursor molecules. Hence, after irradiation, it would be expected that those nuclei which had been prevented from reduplicating their DNA content would be unable to enter mitosis. The net result should be loss, through mitosis, of the Class III nuclei. During the recovery process, DNA synthesis recommences and Class III nuclei should be produced. The DNA-Feulgen data presented in Tables III and IV are in agreement with these ideas. Thus, in the control measurements, Class III nuclei are relatively abundant, whereas shortly after irradiation, they are either absent or very much reduced in number.

It is not possible to determine directly whether or not the first spermatogonia to enter mitosis, after irradiation, are those which, though possessing the requisite amount of chromosomal material, had been prevented from dividing. However, the cytophotometric data strongly suggest that this is the case. The reason is that if such nuclei were prevented from entering mitosis then it should be expected that at 24 hours after irradiation a significant number should be present. The data presented in Tables III and IV show that no Class III nuclei were present in the sample obtained from strain Ba and only two such nuclei (2.4% of the sample) were encountered in the case of strain S.

It might be expected that at 28 days after irradiation, when the frequency of spermatogonial nuclei is much higher than in the controls, that a large proportion of these nuclei should prove to belong to Class III. However, the dry weights of irradiated testes at this time comprise only 34–47% of the control weights. These facts, together with the photometric data, may be taken to suggest that the high rate of mitosis is of a compensating nature and that the spermatogonia enter mitosis immediately after the reduplication of the chromatin is completed.

As shown by Bullough (1948), mitotic activity in the mouse testis remains about constant and at a high level throughout the day. According to the previously mentioned tracer studies, irradiation should bring about a marked decline in the mitotic rate of the seminiferous epithelium (and hence in the area of the tubules occupied by mitotic figures). Reference to Table I and Figures 1 and 3 indicates that at one hour after irradiation, the area of the tubules occupied by dividing spermatogonia is not very different from the control value in the case of strain S, and somewhat lower in the case of strain Ba. By 8 hours, the level has fallen to about 27% of the control in the case of strain Ba, and to 12% in strain S. However, at the present time one cannot rule out the possibility that the mitotic activity has undergone a more rapid decline prior to 8 hours, by which time some regeneration is underway. That this is the case might, perhaps, receive some support from the findings that the one-day values show a substantial increase over the 8-hour levels (from 26.8% of the control value at 8 hours to 65.9% of the control value at 24 hours, or an increase of 140%, in the case of strain Ba; from 11.5% to 21.5%, or an increase of 87%, in the case of strain S).

The duration of mitotic delay (temporary inhibition) as a function of dose has been investigated by Canti and Spear (1929), Henshaw (1932, 1940) and others. (See Lea, 1955, for a discussion of this point.) While the period of delay, for a given dose, varies with the different organisms used, the findings allow the generalization that, for 300 r, recovery would be expected to commence at about 6–8 hours after irradiation. The results reported here for the mouse testis are in fair agreement with this estimate.

The stage at which cells are most sensitive to irradiation has been investigated by Spear (1935), Carlson (1940, 1941, 1954), Creighton (1941) and others. Creighton (1941) has reported the most sensitive stage for grasshopper spermatogonia to be three hours prior to anaphase, that is, at late interphase or early in prophase. The results of Carlson (1941), working with grasshopper neuroblasts, indicate the critical stage to be in prophase, while Spear (1935) concludes from his tissue-culture experiments that late interphase is the most sensitive stage. The work of Henshaw (1940) and others on *Arbacia* eggs suggests prophase to be the most sensitive stage.

The differences noted above are probably not fundamental, but merely represent differences due to the conditions of the experiments in question. The main point is that the most sensitive stage appears to be that part of the mitotic cycle lasting from late interphase to early prophase. Irradiation at or immediately prior to this stage causes the cells to be held up, the duration of this mitotic delay being dependent upon the dose received.

At the time of irradiation, the further away a cell is from this critical stage in its development, the less it is retarded. Similarly, cells which have progressed beyond this stage, at the time of irradiation, do not undergo inhibition of mitosis. These observations afford a partial explanation for the present findings—that the rise in the mitotic rate during the period of 8–24 hours after x-rays is followed by a further and more extensive decline.

In the testis, blockage of spermatogonial mitoses would be expected to result in maturation depletion of the tubules since the spermatogonial cells would be unable to undergo the necessary divisions to renew the proportion of cell types lost (through maturation). The present results confirm this view.

The suppression of DNA synthesis produced by irradiation appears to be induced in cells which were irradiated in interphase since cytophotometric investigations by various workers (see for instance Swift, 1950 and Bryan, 1951) indicate that reduplication of the DNA content, in preparation for the next division, takes place during this phase of the mitotic cycle. Further support for this conclusion may be drawn from the work of Gentcheff and Gustafsson (1939). The findings of these authors suggest that irradiation-induced inhibition of mitosis is caused by the prevention or delay of chromosomal reduplication and consequently the sensitivity of cells to x-rays is correlated with the time of chromosome reproduction.

It is evident that irradiation of cells and tissues brings about specific effects at different levels of organization. Thus at the molecular level there are, for example, such effects as the denaturation of SH-group-containing proteins, and disturbances of the systems responsible for the synthesis of the nucleic acids. At the cytological level, diverse chromosomal observations are encountered as well as effects resulting in the prolongation of prophase (or late interphase). Then there appear to be various latent cellular effects which become evident at a later date—in some instances one or more cell generations later.

It should be expected that a number of cells would receive lethal doses of radiation resulting in immediate death or death prior to the completion of a division cycle. It then follows that if sufficient spermatogonia are destroyed in this manner, maturation depletion of the tubules would ensue. The data of Oakberg (1955) strongly support this hypothesis. However in the present work, as can be seen from Table II, the proportion of necrotic spermatogonia constitutes only a minor fraction of the total. This work therefore supports the alternative hypothesis; that is, irradiation-induced maturation depletion of the seminiferous epithelium is brought about mainly through the irradiation-induced changes responsible for the inhibition of mitotic activity, with cell death playing only a minor role in this process. These conclusions, based on histological and cytophotometric data, therefore do not support the findings of Oakberg (1955), but instead, offer further confirmation of the findings of Eschenbrenner and Miller (1950).

In conclusion, then, the data presented in this paper, with respect to irradiation-induced maturation depletion of the seminiferous tubules, do not support the spermatogonial cell death hypothesis. Instead the data are consistent with the view that the major effects of irradiation are (a) the suppression of mitosis due to inhibition of DNA synthesis and (b) the suppression of mitosis due to the prolongation of prophase through the disturbance of other physiological and biochemical processes necessary for normal mitotic activity.

SUMMARY

1. Changes in the seminiferous epithelium, induced by 320 r of x-rays, have been analyzed by means of a quantitative histological procedure.

2. With the aid of a cytophotometer, measurements have been made of the DNA-Feulgen content of control and irradiated spermatogonial nuclei.

3. The histological data indicate that irradiation, with 320 r of x-rays, results in a temporary maturation depletion of the seminiferous epithelium. This depletion is brought about through the inhibition of spermatogonial mitosis rather than irradiation-induced spermatogonial necrosis. The frequency of spermatogonia reaches its lowest point by three days after irradiation and regeneration does not begin until after 10 days.

4. The cytophotometric data suggest that irradiation-induced inhibition of DNA synthesis is a contributing factor with respect to the suppression of mitotic activity.

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THE ACROSOME FILAMENT AND SPERM ENTRY IN THYONE BRIAREUS (HOLOTHURIA) AND ASTERIAS[1]

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In a number of sea urchin, starfish and mollusc species, Dan (1952, 1954a and 1954b) and Dan and Wada (1955) have demonstrated that the spermatozoon undergoes a profound reaction when treated in one or another of the following ways: exposure to egg water, alkaline sea water, or the presence of eggs; contact with surfaces such as that of glass, a collodion membrane or the egg. The salient feature of this reaction is the formation of a filament, the *acrosome filament,* which extends from the acrosome region. Among other changes is an altered relationship of principal structures. In the echinoderm species the middle piece tends to become displaced to an excentric position and the base of the flagellum then appears to emerge from between middle piece and head, forming almost a right angle with the long axis of the spermatozoon and hence with the projecting acrosome filament.

Spermatozoa having the acrosome filament and structural relationships of reacted specimens described by Dan have also been observed in *Holothuria atra* (Colwin and Colwin, 1955a), in suspensions of sperm treated with egg water or placed in the presence of eggs. In this species it was demonstrated not only that the acrosome filament becomes associated with the egg proper but also that the filament enters the egg intact, as an integral part of the spermatozoon.

The present paper will show that the spermatozoa of another holothurian, *Thyone briareus,* produce acrosome filaments of striking length when appropriately stimulated. The data are, in addition, quantitative. The behavior of the acrosome filament during sperm entry will be described. Collateral confirmatory information on two species of starfish, *Asterias forbesii* and *Asterias vulgaris,* will be included. Abstracts of some of these observations have appeared previously (Colwin and Colwin, 1955b and 1955c).

MATERIALS AND METHODS

Thyone briareus. Specimens were kept in running sea water. Eggs and spermatozoa were obtained from 15 females and 18 males which were eviscerated at intervals between May 13th and June 19th, and from 6 females and 6 males which shed naturally between June 16th and 20th, 1955. Evisceration, a common response in *Thyone* (Killie, 1939), was induced by electrical stimulation of individual animals; then when the gonadal areas were compressed by hand, the gonads were shed along with the other organs. All gonads were rinsed at once in filtered

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[1] Appreciation is expressed for laboratory facilities during the summer of 1955, provided under a contract of the Office of Naval Research with The Marine Biological Laboratory.

sea water. The testes were then stored "dry." To obtain sperm the tubules were cut once or twice with scissors. The sperm which oozed out was then suspended in filtered sea water and variously diluted. No differences were detected between the active spermatozoa obtained in this manner and those shed naturally. Eggs were gently stripped through the cut ends of individual ovarian tubules. A thin cellular capsule, which did not persist on naturally shed eggs, enclosed the stripped egg and its surrounding jelly. Frequently this capsule could be completely or partially removed by sucking the eggs into a glass syringe through a hypodermic needle of No. 27 bore. Then the needle was detached and the eggs slowly expelled into filtered sea water. Effective egg water was procured from these decapsulated stripped eggs as well as from eggs which had been shed naturally.

The egg of *Thyone* is flattened axially. It appears elliptical in lateral view and somewhat circular in polar view; the diameter of this circle is approximately 250 μ. Near the animal pole a typical holothurian "umbilicus" projects from the egg into the surrounding jelly. The jelly hull is approximately 55 μ thick and contains radial striations, some extending as far as its outer edge. These irregular wavy striations differ markedly from the clearly-defined rigid looking acrosome filaments of entering spermatozoa.

Sections of naturally shed eggs show that the germinal vesicle is not intact, confirming the findings of Ohshima (1925). In the living egg, however, the vesicle often *appears* to remain intact owing probably to persistence of its "residual substance," as noted by Ohshima. Eggs from eviscerated gonads were not sectioned.

Asterias. Germ cells of *A. forbesii* were obtained from freshly collected specimens in May and June, 1955. The gonads were removed from detached arms. Ovaries placed in large dishes of sea water shed copiously. Fresh eggs were removed, rinsed several times and put into large dishes of sea water. Concentrated sperm, collected from testes which had been stored "dry," was suspended and diluted in filtered sea water as needed. A few specimens of *A. vulgaris,* observed on May 14th, were also handled in the manner just described.

Alkaline sea water was prepared by adding sufficient 0.1 N NH_4OH to freshly ~~diluted~~ filtered sea water to bring it to pH 9.2–9.4, as determined by color comparison with thymol blue. In some cases a slightly higher pH was used. The sperm suspensions were then added to this alkaline sea water. The actual pH of these final mixtures was not determined but would obviously be lower than that of the original alkaline sea water.

All photographs and sketches shown were made from living material as viewed with oil immersion objectives. Both bright field and phase contrast microscopes were used.

Observations

I. The acrosome filament

A. *Thyone briareus*

1. *Formation of acrosome filament in treated sperm suspensions.* Spermatozoa were treated with either *egg water* or *alkaline* (*ammoniated*) *sea water.* Drops of sea water-suspended sperm were added to drops of either solution and, for controls, to filtered sea water in the same proportions. Some typical results as found in

living preparations are shown in Table I. In all these experiments appropriate treatment of the sperm resulted in the production of acrosome filaments in percentages significantly greater than the controls. The alkaline sea water was a much more effective stimulant than the egg water within the concentrations of the latter used. This is shown strikingly in experiments 6 and 7 in which in each case one original sperm suspension was used with the egg water and the alkaline sea water; in both experiments the egg water was ineffective (too weak?) whereas the alkaline sea water caused 90% or more of the spermatozoa to form acrosome filaments.

TABLE I

Acrosome reaction of spermatozoa of Thyone briareus. Production of acrosome filaments evoked by adding sea water sperm suspensions to egg water or to sea water made alkaline by addition of $0.1\ N\ NH_4OH$

Number of experiment	Number spermatozoa counted	% with filaments	Number spermatozoa counted	% with filaments
	Egg water		sea water control	
†EG, SN-1	100	21	100	2
EG, SN-2	52	21	>100	0
EG, SG-3	100	18	100	3
EN, SN-4	104	12.6	100	1
EG, SG-5	73	6.8	100	0
EG, SG-6(a)	111	1.8	††sweep	0
EG, SG-7(a)	100	0	sweep	<1
	Alkaline sea water		sea water control	
EG, SG-6(b)	sweep	>90*	sweep	0
EG, SG-7(b)	sweep	>99*	sweep	<1
EG, SG-7(c)	97	92**	sweep	<1
EG, SG-8	sweep	>90**	sweep	0

Experiments bearing the same number were made simultaneously from the same sperm suspension and have the same control.

† E, eggs; S, sperm; N, naturally shed; G, obtained by removal from gonad.

†† Sweep: extensive examination of area of coverslip by back and forth movement with a mechanical stage.

* pH of alkaline sea water to which sperm was added was slightly higher than 9.2–9.4.

** pH of alkaline sea water to which sperm was added was 9.2–9.4.

2. *Acrosome filaments in untreated sperm suspensions.* Examination of the control figures of Table I makes it clear that spermatozoa with acrosome filaments are sometimes found in a very low percentage in untreated sperm suspensions. Perhaps these result from "contact" stimulation, as Dan has suggested for other species, or from as yet unknown factors.

3. *Acrosome filaments in inseminated cultures of eggs.* The acrosome filament is always found on spermatozoa which are entering eggs. It is also seen on spermatozoa which lie in the jelly but fail to enter the egg; it may (Figs. 2, a and 8) or may not extend as far as the egg surface; its position in the jelly is usually radial, sometimes tangential and occasionally even directed away from the egg surface.

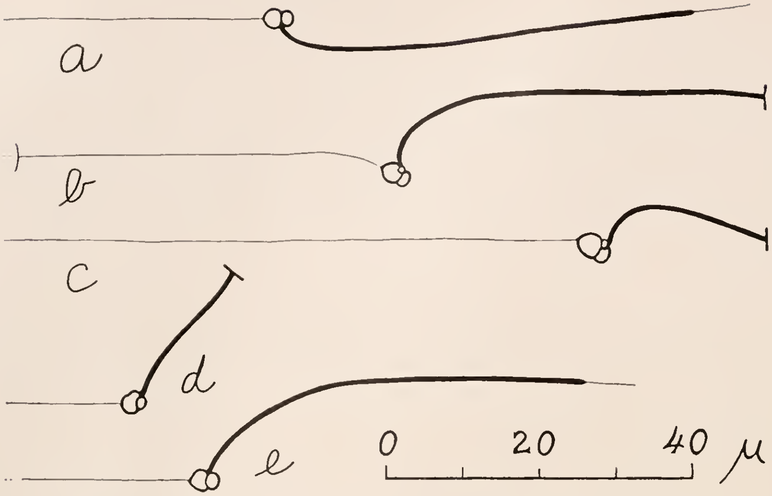


FIGURE 1. Reacted spermatozoa showing acrosome filament, from photographs of living specimens. a–c, *Thyone briareus;* d–e, *Asterias forbesii.* a, in egg water (35 μ); b, at egg surface (48 μ); c, in alkaline sea water (75 μ) (position of distal part of flagellum modified for reasons of space); d, in inseminated culture but directed away from egg (15 μ); e, at egg surface (22 μ).

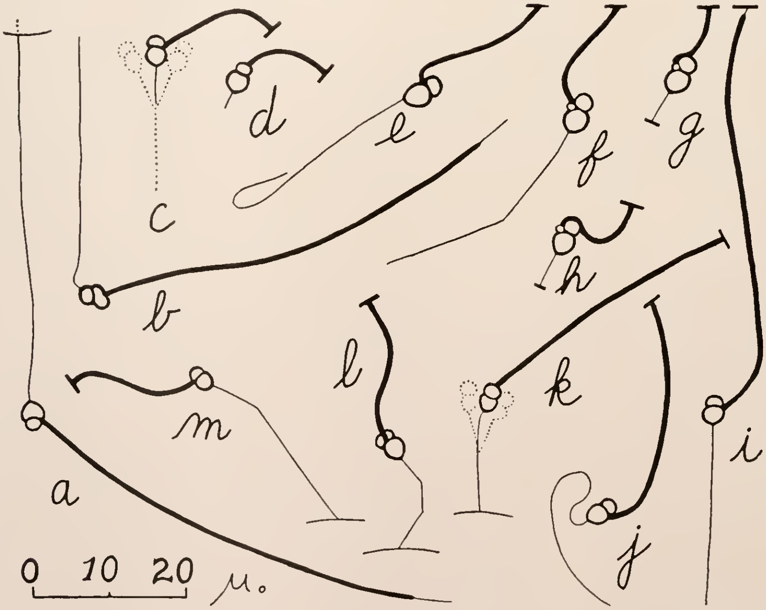


FIGURE 2. Reacted spermatozoa: bending and curving of acrosome filament. From sketches and photographs of living specimens. a–h, *Thyone briareus;* i–l, *Asterias forbesii;* m, *A. vulgaris.* a, at egg (presumed distal portion in dotted line); b, in egg water; c–h, in alkaline sea water. c, oscillating about fixed point (presumed distal part of acrosome filament dotted); d, same specimen as in c, after separating from fixed point; g–h, successive positions of flagellum shown in f; i–j, successive views of one specimen, in inseminated culture but not at egg; k–m, at surface of eggs (k, oscillating specimen).

Spermatozoa with acrosome filaments are also found near but entirely outside the jelly hull of the egg; in these no particular orientation with reference to the egg has been noted.

4. *Appearance of the unreacted spermatozoon.* Fresh sea water suspensions of sperm were spread thinly between slide and coverslip and individual spermatozoa examined as soon as they became sufficiently quiet. As seen in outline (Figs. 3, a–c and 5) the head is nearly circular. Closely applied to it is a narrow curved structure containing the middle piece and, apparently, a highly refringent circular body. The flagellum projects posteriorly as though from the middle piece but its actual point of origin is obscure. The flagellum is about 60 μ in length; its terminal portion or end piece is about 6 μ and much thinner than the main portion. The rounded acrosome, somewhat subterminal to the anterior part of the head, is often not seen, presumably because of the position of the specimen; it seems to intrude into the mass of the head (Fig. 3, a–c).

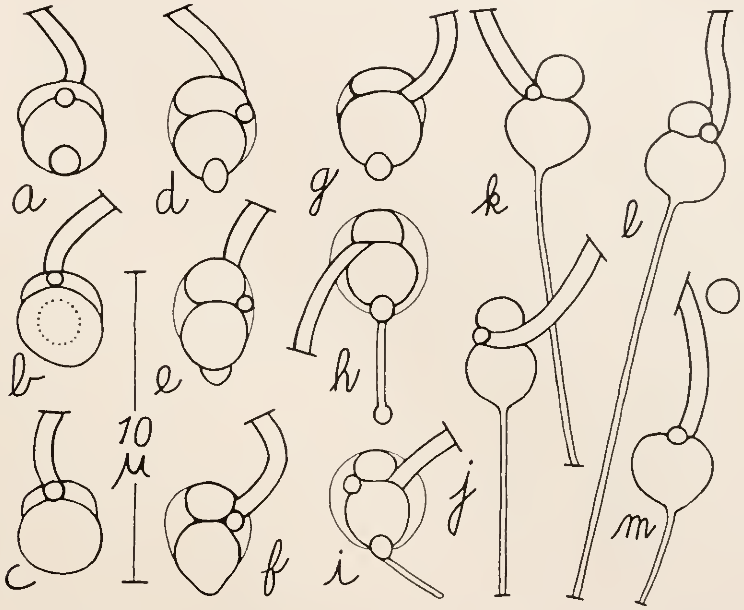
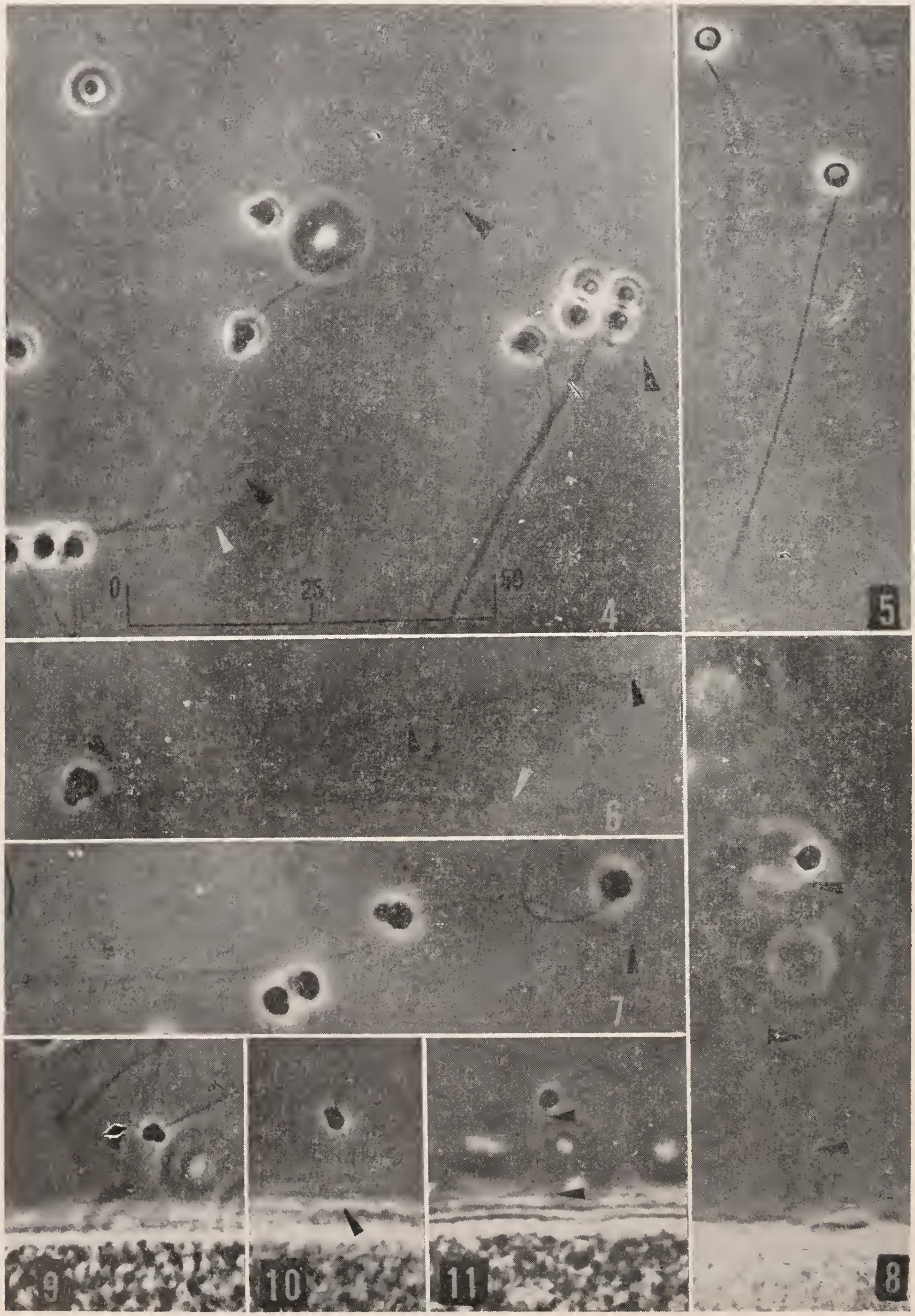


FIGURE 3. Spermatozoa of *Thyone briareus,* from sketches and photographs of living specimens. a–c, unreacted, in sea water; d–e, moribund, in aging sea water suspensions; f–g, partial reaction (?), in inseminated culture but not at egg; h–i, partial reaction (?), in alkaline sea water; j–l, reacted, as seen in all media described in text; m, reacted, entering egg, middle piece separated as seen occasionally.

5. *Appearance of the reacted spermatozoon.* Reacted spermatozoa are morphologically indistinguishable from each other, whether they occur in egg water (Figs. 1, a and 4), in alkaline sea water (Figs. 1, c and 6–7), in association with the egg (Figs. 1, b and 8), or in untreated sperm suspensions; they are, however, markedly different from unreacted spermatozoa. The middle piece is round in outline, forming with the head the configuration of a figure eight (Figs. 1, a–c and 3, j–l). The flagellum emerges posterolaterally from between them, com-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FIGURES 4–11.

monly seeming to arise from the head (Figs. 1, a–c and 3, k–l). The refringent circular body lies near the point of contact between head and middle piece (Fig. 13). Anteriorly, from the acrosome region, projects the acrosome filament, frequently at an approximate right angle with the base of the emerging flagellum (Figs. 1, a–c and 5).

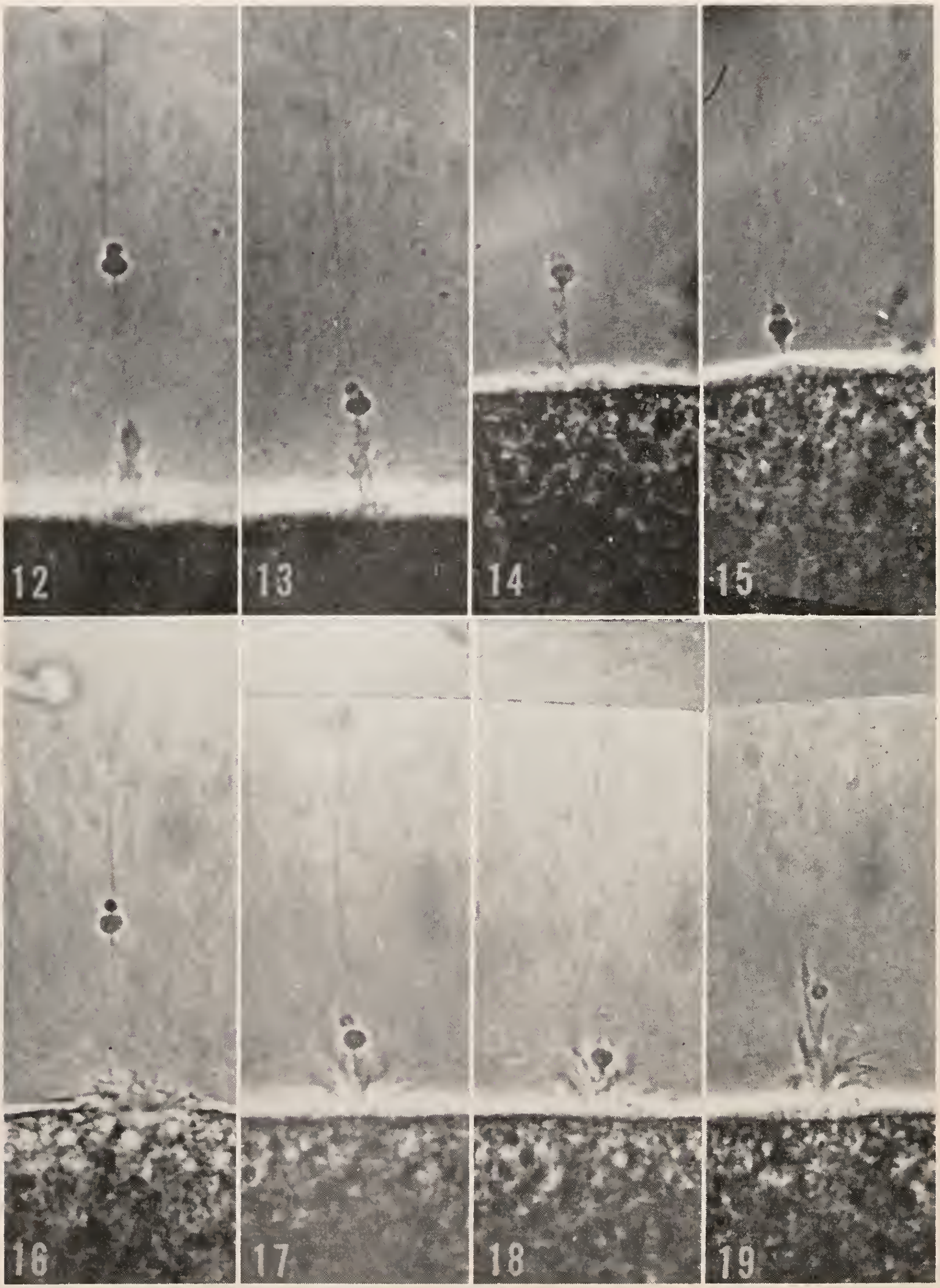
Successive stages of a specific reacting specimen have not been observed. Under certain conditions individuals are found like those shown in Figure 3, d–e (from aging sperm suspensions), Figure 3, f–g (in inseminated cultures) and Figure 3, h–i (in alkaline sea water). Here the enclosing membrane is loosened; the head, middle piece and refringent body are rounded and clearly discrete; and the flagellum emerges laterally, much as reported by Dan (1954a) for moribund spermatozoa of starfishes. The acrosome, however, is an additional balloon-like structure (*e.g.*, in Fig. 3, d) whose contents, under phase contrast, appear pale and diffuse as compared with the darker, seemingly more dense, head. Occasionally there is a short acrosome filament (Fig. 3, i) which is sometimes slightly thicker and ends in a rounded knob (Fig. 3, h). Though enclosing membranes are not prominent in fresh specimens, whether reacted or not (Figs. 3, a–c and j–l), it may be that these spermatozoa with loosened membranes hold clues to the intermediate stages of the acrosome reaction; except for the appearance of the membranes, Figures 3, g–i, for example, might be transitions from Figure 3, a to Figure 3, l.

6. *Dimensions of acrosome filament.* It has not been ascertained what length characterizes the average, fully reacted acrosome filament but most of those examined ranged between 35 μ and 65 μ. Several spermatozoa like the one shown in Figure 8 (which had attached but failed to enter the egg) had filaments measuring 46–48 μ. The acrosome filament of the spermatozoon shown in Figure 6 (and schematically in Fig. 1, c) measured 75 μ and exceeded the length of the flagellum. In one exceptional case the filament measured 90 μ. The acrosome filament is extremely tenuous, its diameter being of the general order of magnitude of that of the end piece of the flagellum.

B. *Asterias forbesii and Asterias vulgaris*

In both species reacted spermatozoa were found in fresh living preparations of inseminated eggs. Except that their acrosome filaments were very much shorter, the reacted spermatozoa of both species looked very much like those of *Thyone*. The heads were somewhat smaller but the flagella measured about 55–60 μ in length, including the short filamentous end piece. In *A. forbesii* the maximum length of the acrosome filament was approximately 25 μ; acrosome filaments attached to but failing to enter eggs (Figs. 1, e, 10 and 11) measured from 15–22 μ. The same order of magnitude prevailed in *A. vulgaris* (Fig. 2, m). In both species some reacted spermatozoa were found with their acrosome filaments directed *away* from the eggs (Figs. 1, d and 9).

FIGURES 4–11. Unretouched photographs of living spermatozoa; all to scale shown in Figure 4, representing 50 μ. Black arrows point to acrosome filament, white arrows to end piece of flagellum. Figures 4–8, *Thyone briareus;* Figures 9–11, *Asterias forbesii.* All figures show reacted spermatozoa with acrosome filament except Figure 5, which shows unreacted specimens. Figure 4, in egg water; Figures 6–7, in alkaline sea water; Figures 8, 10–11, at surfaces of eggs but did not subsequently enter; Figure 9, near egg but directed away from it.



FIGURES 12–19. Unretouched photographs of living spermatozoa penetrating egg of *Thyone briareus*. To same scale as in Figure 4. (All Figures except 15 and 16 have had non-relevant pieces spliced in to upper portions.) Figures 12–13, successive views of slender cone with broad

C. *General nature of the acrosome filament*

Some properties of the acrosome filament may now be described. As they are apparently much the same in both *Thyone* and *Asterias,* and whether they occur in egg water, alkaline sea water or in inseminated cultures, reference to species and medium will be omitted here but may be found with the appropriate illustration. Despite its thread-like dimensions the filament is usually straight (Figs. 2, i, 4, 6 and 11) or curved only in very wide arcs (Figs. 2, a and 8). Figure 2, k shows a specimen which was attached to (but did not enter) an egg; the filament curved slightly as the head oscillated with the motion of the flagellum. When not at the egg the distal portion and/or tip of the filament often sticks to the slide or cover-slip; for example, the specimen shown in Figure 2, i–j, moved suddenly, causing the non-adherent proximal part of the filament to curve sharply. Other partly adhering, sharply curved filaments are shown in Figures 2, b and 2, e. The acrosome filament *may* even break. This could explain the spermatozoon shown in Figure 2,

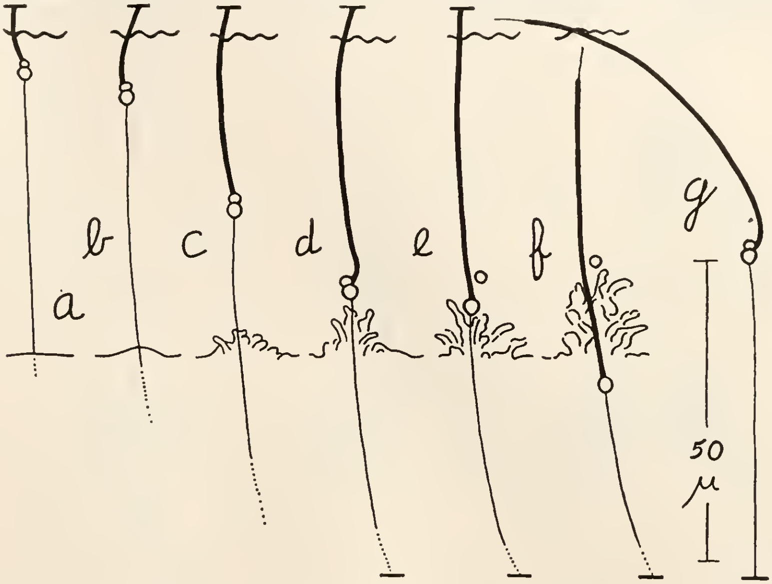


FIGURE 20. a–f, scheme of sperm entry process in *Thyone briareus;* part shown in dotted line represents minimum additional length of acrosome filament presumed but not actually seen to enter egg. g, reacted spermatozoon from alkaline sea water suspension for comparison of length of acrosome filament. a, presumed very early stage based on spermatozoon which failed subsequently to enter; b, low broad incipient cone rises as acrosome filament proceeds into egg proper; c–f, successive stages of sperm entry, acrosome filament enters egg proper as advance integral part of spermatozoon (middle piece, left outside in this case, frequently enters egg). In subsequent stages, entire flagellum passes into egg proper.

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base partly retreated; note acrosome filament in cone. Figures 14–15, successive views of one specimen, very slender cone; sperm head did not subsequently enter egg proper. Figures 16–19, successive views of specimen in which low broad cone gave rise to filose projections which continued growing outward even after sperm head had entered egg proper; no tall slender cone formed in this specimen; middle piece failed to enter.

c–d; when first seen it was oscillating like a pendulum with a short stem. Suddenly it began to whirl freely in many directions, and its filament seemed even shorter; the presumed adhering distal part of the filament was not observed. A number of spermatozoa have been seen with the filament bent permanently (Figs. 2, f, l, m, 7 and 10).

II. Sperm entry

A. *Thyone briareus*

Many individual eggs were examined, both from natural sheddings and stripped from the gonads. As lightly inseminated cultures of the naturally shed eggs gave 98–99% cleavage and more than 90% active larvae, it was evident that these were normal eggs. On the other hand, cleavage was never observed in inseminated cultures of the stripped eggs. Yet, whichever the source, a number of spermatozoa entered every egg which was observed individually in slide and coverslip preparations. Generally, the entry was more rapid and the height of the cone less great in the naturally shed eggs.

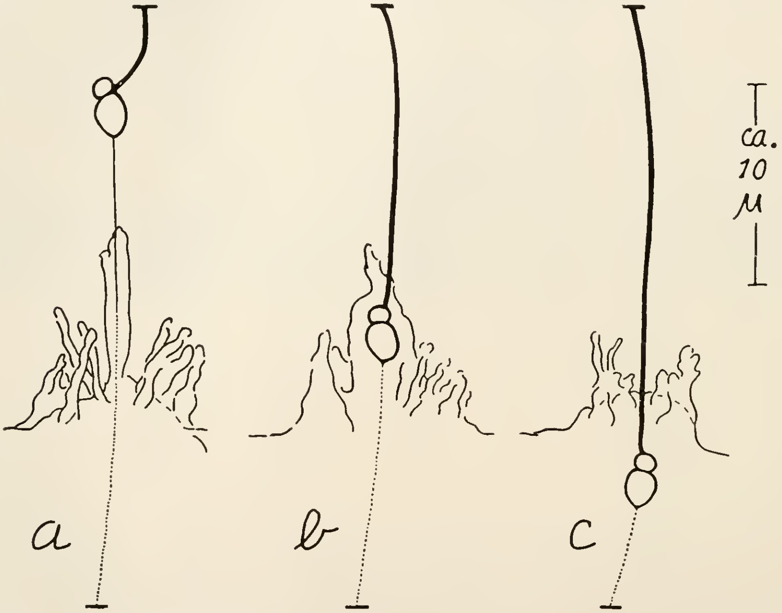

FIGURE 21. Successive stages of sperm entry in *Thyone briareus*, from sketches of a living specimen. A broad cone with filose projections elevated one moderately slender sleeve about the inmoving acrosome filament. Acrosome filament seen in outer part of cone: solid line. Presumed further course of filament: dotted line.

A scheme of the entry process is shown in Figure 20. The spermatozoon does not swim through the thick jelly hull; its first contact with the egg surface is made by means of its acrosome filament. Presumably a very early stage would appear as in Figure 20, a. At the earliest stage noted frequently in successful entry, the main body of the spermatozoon had already progressed part way through the jelly and its acrosome filament was already associated with the broad low hyaline incipient cone (Fig. 20, b). In some instances, as the spermatozoon continued moving inward, filose projections arose from the cone and continued growing outward even after the sperm head had passed through the cone into the egg proper (Figs. 16–19).

Some common variations in the cone are shown as they appeared in specific living eggs. The cone shown in Figures 16–19 never rose extensively above the egg surface. Sometimes a moderately slender projection would move outward like a sleeve ascending the inmoving acrosome filament, and then retract after the sperm head had entered the cone, as in Figure 21, a–c. In this specimen the cone remained broad at the base throughout the entry process but often an initially broad base would retreat partly or wholly so that the only obvious external cone would be a single slender projection (Figs. 12–13, 14 and 22, a, c and e). Transitory changing dilations appeared along such cones (Figs. 12–13 and 14). The cones were sometimes so slender that they might easily have been called "filaments" by an observer not cognizant of the existence of the much more delicate acrosome filaments (Figs. 14, 22, a and e). There was also variation in the distance from the egg proper at which the sperm head entered the cone (*cf.* Figs. 22, b and d). In the specimen shown in Figure 22, c–d a column of hyaline protoplasm continued to grow outward for some time after the sperm head had passed into the egg proper.

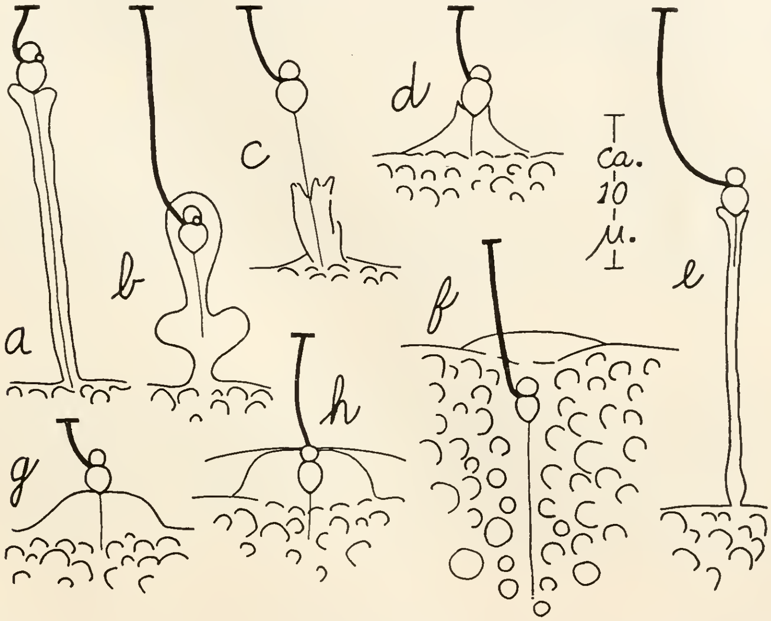

FIGURE 22. Variations in sperm entry, from sketches of living specimens. a–f, *Thyone briareus;* g–h, *Asterias forbesii.* Acrosome filaments shown only to depth actually seen. a–b and c–d, successive views of two specimens, respectively; e, very slender cone embracing acrosome filament; f, acrosome filament within egg proper; g–h, from two specimens of *A. forbesii,* successive stages which preceded stage closely resembling that shown in f.

Although the middle piece ordinarily entered the egg with the sperm head (Figs. 21, 22, b and f) it sometimes became separated (Fig. 3, m) and remained outside altogether (Fig. 19). Occasionally an apparently detached middle piece eventually passed into the egg. Several times a detached middle piece was seen to vibrate vigorously with the movement of the flagellum while the head continued moving on into the egg without pronounced vibration.

The entire flagellum passes into the egg. However, it was sometimes not seen or seen only with difficulty within the cone, even in cases in which it was observed continuously until finally the end piece disappeared into the cone.

The much more tenuous acrosome filament was also seen only with difficulty after it had been enveloped by the cone (Figs. 12–14 and 22, c–d). Sometimes only a portion of the filament could be seen (Fig. 22, e). In other cases a part of the filament would be seen and then disappear with the changing condition of the cone (Figs. 22, a–b). Within the egg proper, observation was further hindered by the presence of yolk granules (Fig. 15) and it was only in some specimens that the acrosome filament was seen there with certainty (Fig. 22, f). Occasionally some disturbance of the yolk granules in advance of the entering sperm head suggested the presence of an acrosome filament not actually seen. In the scheme of sperm entry shown in Figure 20 the acrosome filament has been represented by solid lines to the depth within the egg that it has sometimes been seen, and in dotted lines to the depth to which it might be presumed to penetrate, judging by the known lengths of acrosome filaments as seen in alkaline sea water (Fig. 20, g) or attached to but not entering the egg (Fig. 20, a).

B. *Asterias*

A small number of polyspermic eggs of *A. forbesii* were examined. In the earliest stages observed the spermatozoa were already quite close to the egg surface. In some cases the acrosome filament was seen within the cone (Fig. 22, g); in a few it extended into the protoplasm of the egg proper (Fig. 22, h). Several times an appreciable length of the filament was seen within the egg, very much as shown in Figure 22, f representing *Thyone*.

Discussion

A. Acrosome filaments

The acrosome reaction resulting in the production of a filamentous structure, the acrosome filament, first demonstrated by Dan, has been shown here to occur also in the holothurian, *Thyone briareus*. Whereas the longest previously reported acrosome filament, that of starfishes (Dan, 1954a), measured 22–28 μ, the acrosome filament of *Thyone* may reach two to three times this length and can even exceed the length of the flagellum.

Recently Rothschild and Tyler (1955) have questioned that the acrosome filament is formed by means of an acrosome reaction, in the sense of Dan. Their reservations are based on observations of untreated sperm suspensions of two species of mollusc and two species of sea urchin, viewed by both phase contrast and electron microscopy. They state that in such preparations generally two types of spermatozoa are found, one type with a short "acrosomal filament" and one with a long "acrosomal filament." Two such types of sperm are shown, for example, for the sea urchin *Echinocardium cordatum* in their Figures 8 and 9. Their Figure 8 shows a spermatozoon with a fairly long and knobbed acrosome, and it appears as previously described for this species by Vasseur (1947) and by Afzelius (1955); it was the form invariably found in a living sperm suspension. Their Figure 9, however, shows a spermatozoon with a profoundly different appearance; a filament much longer than that of the other type extends from the apex of the head, the middle piece is excentric, and the flagellum and filament lie nearly at right angles to each other. In all these features the spermatozoon resembles the reacted spermatozoon of the sea urchin as described by Dan (1952). Furthermore, Tyler (1952) has previously reported that fertilizin-treated sea urchin spermatozoa show just such a displacement of the middle piece from the normal position to a postero-lateral one. There seems little doubt, then, that the spermatozoon shown by Rothschild and Tyler in their Figure 9 represents a reacted spermatozoon and the long filament extending from the apex of the head is an acrosome filament, in the sense of Dan.

Perhaps the reservations of Rothschild and Tyler might have been obviated by the presence of quantitative data in the reports by Dan; and similarly it would be very interesting if Rothschild and Tyler had presented quantitative data of *treated* sperm suspensions to compare with their results of the untreated suspensions. Any reservation regarding the existence of the phenomenon of the acrosome reaction should be dissipated by the quantitative data presented here for *Thyone,* in which it is clearly demonstrated that the treated preparations show significantly greater percentages of reacted spermatozoa with acrosome filaments than untreated preparations, *as observed exclusively in living material.* The tremendously long acrosome filaments of *Thyone* make these observations relatively easy.

The occurrence of the two types of spermatozoa observed by Rothschild and Tyler in their untreated preparations may have been caused by "contact" or other as yet unknown factors which supervene during preparation of the sperm suspensions. It is important to note that in *Echinocardium cordatum* Rothschild and Tyler found the long "acrosomal filaments" only in *fixed* material; the possibility exists that in this case the fixative, or some feature of the process en route to fixation, may have been the stimulus for the acrosome reaction. In this connection an observation of Lillie (1912) on *Nereis* now becomes extremely interesting; he noted that in the *living* spermatozoon the perforatorium (acrosome) was shaped like the spike of a helmet and was *shorter* than in the *fixed* spermatozoon, whether the latter was free or attached to an egg; in this fixed material the acrosome had the shape of a filament. What Lillie observed in the fixed material would seem to be an acrosome filament resulting from an acrosome reaction (possibly caused by fixation). Recently Metz and Morrill (1955) have also studied fixed spermatozoa of *Nereis;* the acrosome filament was present in low percentage in normal sea water controls, and in significantly higher percentage in spermatozoa which had been treated with fertilizin. A more detailed discussion of this and related matters is presented elsewhere (Colwin and Colwin, 1956).

B. Sperm entry

In *Holothuria atra* (Colwin and Colwin, 1955a) the acrosome filament behaves as an integral part of the sperm head, enters the egg intact and remains not appreciably altered until at least well within the egg proper. A relatively broad cone rises to surround the entering filament like a sleeve. Frequently a short narrow projection creeps up the acrosome filament in advance of the main body of the cone. In *Thyone,* although the cones are narrower, and the acrosome filaments a great deal longer, the major aspects of sperm entry are much the same, namely, the acrosome filament enters the egg as an integral part of the spermatozoon and remains essentially intact at least during its initial entry into the egg proper; the substance of the cone moves outward enclosing *the acrosome filament which has already established the first true contact between spermatozoon and egg proper.* But whereas in *H. atra* all the observations were made on eggs which had been stripped from the gonads artificially, in *Thyone* not only stripped eggs but also normally shed eggs, controls of which developed normally, showed essentially the same picture. This strengthens the suggestion, made previously, that sperm entry as described for *H. atra* reflects the essentials of the entry phenomenon in the normal egg of that species, particularly with respect to the behavior of the acrosome filament.

The tall slender type of cone usually seen in *Thyone* strongly suggests the reception cone described by Hörstadius in *Holothuria poli* (1939a) and the starfish *Astropecten aranciacus* (1939b). In *Thyone,* however, since the cones can be seen to embrace the already established inmoving acrosome filament, there is no basis for their interpretation as the agents of first contact between egg and spermatozoon, as has sometimes been held. A more extensive discussion of the mechanism of sperm entry and a reinterpretation of the events of entry as described in echinoderms by earlier workers will be found elsewhere (Colwin and Colwin, 1955a and 1956).

From the measurements in the present observations it is evident that the acrosome reaction in *Thyone* is capable of producing acrosome filaments much longer than the depth of the jelly hull of the egg. The question arises: how long must the acrosome filament be in order to initiate successful sperm entry? As the spermatozoon does not swim through the jelly in *Thyone,* it seems that the minimum length necessary would be that of the thickness of the jelly.

The authors have never witnessed a subsequently entering spermatozoon at the exact moment of its first contact with the egg in *Thyone,* or indeed in *H. atra* and the three species of *Asterias* which have been studied. In all of these it has been considered likely that the earliest stage would resemble that seen when spermatozoa attach to the egg but fail subsequently to enter, as in Figures 8, 11 and 20, a. However, since it is known that the acrosome filament of *Thyone* can be longer than in such attached spermatozoa, the possibility certainly exists that these attached specimens represent a stage later than the earliest one. Inasmuch as the filament of successfully entering spermatozoa moves into the egg, perhaps in these attached non-entering spermatozoa some distal portion of the filament may already extend into the egg.

SUMMARY

1. As shown by quantitative data, spermatozoa of *Thyone briareus* treated with egg water or alkaline (ammoniated) sea water undergo an acrosome reaction resulting in the production of acrosome filaments in a percentage significantly greater than found in untreated controls. Acrosome filaments are also found in inseminated cultures of eggs, either associated or unassociated with the eggs.

2. The acrosome filaments of *Thyone briareus* are exceptionally long and may even exceed the thickness of the jelly hull (55 μ) or even the length of the flagellum (60 μ).

3. Reacted spermatozoa of *Asterias forbesii* and *A. vulgaris* seen in inseminated cultures of eggs have acrosome filaments measuring about 15–22 μ.

4. The general nature of the acrosome filament is much the same in both species of *Asterias* and in *Thyone,* regardless of the stimulating agent. Though thread-like in dimensions it is usually straight, or curved only in a wide arc. However, it is capable of curving sharply, bending and perhaps even breaking. The tip or distal portion often sticks to the glass of the slide.

5. At sperm entry in *Thyone* and *Asterias* the acrosome filament makes the initial contact with the egg and then enters the egg as the first element of the spermatozoon of which it is an integral part. In *Thyone* the cone is sometimes so slender as to appear filamentous itself, even though it contains the acrosome filament.

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POLYPHENOL OXIDASE ACTIVITY AND PIGMENTATION IN SNAIL TISSUES[1]

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Relatively little has been done to elucidate the mechanisms of intermediary metabolism in molluscs despite their recognized medical and economic importance (von Brand, 1952). A knowledge of such mechanisms would seem to be an essential preliminary to any rational investigation of these forms as parasite vectors or any logical attempt toward their control. In addition, comparative information concerning metabolically important reactions in different animals is a valuable aid to our understanding of the patterns of animal metabolism in general.

As part of an over-all study of snail physiology and biochemistry various aspects of the functioning of the midgut gland (hepatopancreas) of certain fresh water snails have been investigated. During one phase of this program a study was made of the mechanisms thought to be responsible for the presence of the reasonably large amount of pigment normally found in this gland in the fresh water operculate snail, *Ceratoides cornvarietis* (Linné, 1758). The deposition of melanin pigments in various tissues is a characteristic activity in many organisms, but the function, if any, of such pigments is frequently obscure. Since the pigmentation is the normal result of metabolism in the liver of the snail and since it has been suggested that the oxidative components of the series of chemical transformations resulting in melanin formation may be linked to the electron transfer systems of certain aerobic dehydrogenases (Baldwin, 1952) it was felt that a study of the function of snail liver must include some references to these reactions. The present report deals with some of the characteristics of the polyphenol oxidases in snail liver and their probable connection with melanin formation.

METHODS

The fresh water snail, *Ceratoides cornvarietis,* used in the present study was initially obtained from a stream near Rio Piedras, Puerto Rico. The animals used in the present study were raised in this laboratory from the original stock. This snail is not a vector for any known medically important parasite, but was chosen for the initial studies in snail physiology because of its large size, abundance, hardiness in the laboratory, and, importantly, because of the fact that in this snail, by careful dissection, the gonads could be fairly well separated from the midgut gland. It was expected that the results obtained and techniques developed in the study of this snail would allow a more effective approach to the later study of more medically

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important forms. The snails were kept in the laboratory in constantly aerated pond and well water aquaria. They were not starved (Baldwin, 1938) before experimental use as no significant improvement in the consistency of results was obtained in this manner.

The assays for enzyme activity were carried out on cell-free homogenates (Potter and Elvehjem, 1936) prepared in cold, glass-distilled water from tissue samples carefully dissected from large mature snails. The use of varying salinities as suspending media failed to significantly alter the results. In the preliminary experiments the tissues were segregated according to the sex of the animal, but when no fundamental differences were observed this procedure was abandoned. The homogenates were uniformly centrifuged at low speed (600 × g) to remove the major part of the heavy inert pigment before being diluted to a final 5% concentration. This dilution effectively prevented any oxygen consumption by endogenous metabolism. All of the foregoing procedures were carried out at 4° C. and the reaction flasks were kept thoroughly chilled until placed in the water bath. The reaction mixtures were assayed in Warburg flasks in an atmosphere of air at 25° C.

TABLE I

Per cent dry weight, total nitrogen, and the ether-soluble fraction of snail liver

% dry wt.		N_2 mg./gm. dry wt.		Lipid mg./gm. dry wt.	
Male	Female	Male	Female	Male	Female
27.4	25.6	74.8	68.2	164	230
27.0	28.9	75.2	67.4	144	209
27.0	27.1	72.9	68.2	151	214
28.1	28.2	73.4	69.1	153	198
27.2	26.8	72.8	66.9	167	207
27.0	26.9	72.9	67.0	172	222
26.9	28.1	74.3	68.2	154	214

Suitable controls were included in each run. Initial runs were made to insure optimum conditions in regard to enzyme concentration, substrate concentration, and pH. Dry weight determinations were made to check against differences in individual tissue samples as were total nitrogen determinations as a crude test for total protein (micro-Kjeldahl) and fat determinations (ether-soluble weight loss method).

RESULTS AND DISCUSSION

During the course of the assays for specific enzyme activity periodic checks gave the results on dry weight, total nitrogen, and the ether-soluble fraction which are summarized in Table I. Although both male and female livers have *ca.* 27% dry material, the male liver contained somewhat more nitrogen and less ether-soluble material. In dissecting out the female liver some difficulty was encountered in completely freeing the liver from extraneous tissues. The differences in the nitrogen and fat data between the sexes, therefore, are possibly a reflection of the difference in the closeness of the anatomical relationship of the liver and gonad. Apparently, however, this difference in the tissue samples was not significant from

Table II

Influence of enzyme concentration, substrate concentration (catechol), and pH on the activity of polyphenol oxidase activity in snail liver homogenates. All but the experimental substances were at optimum concentration

Enzyme concentration (mgm. snail liver, wet wt.)			5	10	25	30
Q_{O_2}			26 (23–29)	56 (54–58)	108 (99–115)	111 (101–121)
Substrate concentration (catechol in micromols)			10	20	50	60
Q_{O_2}			25 (23–29)	42 (38–47)	99 (95–110)	103 (98–108)
pH	6.8	7.2	7.6	8.0	8.4	8.8
Q_{O_2}	41 (35–52)	73 (67–79)	92 (86–99)	106 (97–111)	91 (87–96)	62 (53–71)

the standpoint of the enzyme activity, since no variation between sexes in this regard was obtained from the assays.

In Table II are given the results of studies on the influence of enzyme and substrate concentration and the pH on the activity of the snail liver polyphenol (catechol) oxidase activity. Optimum conditions were obtained with a reaction mixture containing 25 mgm., wet weight, of cell-free homogenate, 50 micromols of substrate (catechol), and 0.033 *M*, final, potassium phosphate buffer at a pH of 8.0. The addition of neither methylene blue, diphosphopyridine dinucleotide, nor cytochrome *c* to the reaction mixture to serve as a hydrogen carrier was of any benefit to this system. From this it would seem that the system is an aerobic oxidase rather than an aerobic dehydrogenase. The replacement of the air in the reaction system with 100% oxygen was also of no significance in promoting higher activity. The enzyme was found to exhibit a marked stability for several hours at room temperature and it was still highly active after refrigeration for 96 hours.

Table III

Activity of the monohydric and polyhydric phenol oxidases in snail liver homogenates. Reaction flask contained 25 mgm. (wet weight) liver homogenate, 0.1 M pH 8.0 phosphate buffer, 50 micro. mol. substrate, 1 ml. 20% NaOH in center well. Each figure is the average of closely approximated duplicates

Substrate	Q_{O_2}					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
p-Cresol	0	0	0	0	0	0
Phenol	16	0	0	0	0	0
Tyrosine	0	0	9	0	0	0
Guaiacal	6	0	21	0	0	0
Catechol	104	107	107	106	110	99
Pyrogallol	142	141	144	145	132	134
Adrenaline	72	79	75	69	72	73

In the frozen or lyophilized state it was stable up to six months. In this regard the enzyme resembles the tyrosinase of earlier workers (Lardy, 1949).

However, the enzyme system in snail liver was observed to seemingly possess a generic specificity for polyhydric phenols (Table III). Negligible activity was

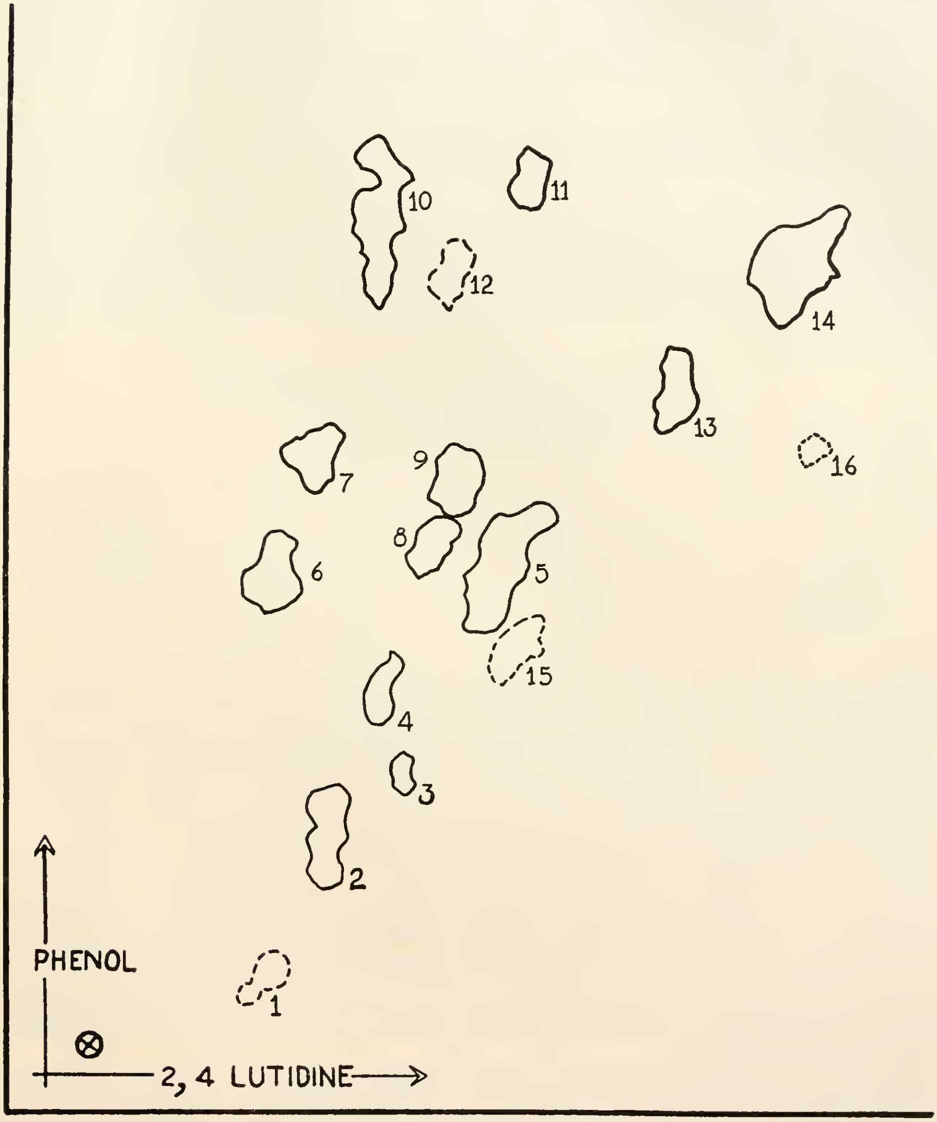

FIGURE 1. Two-dimensional composite chromatogram of the liver from *C. cornvarietis* extracted for free amino acids. Broken lines indicate relatively low concentration. 1—aspartic acid; 2—glutamic acid; 3—serine; 4—glycine; 5—alanine; 6—lysine; 7—arginine; 8—citrulline and/or glutamine; 9—β-alanine; 10—unidentified polypeptid; 11—proline; 12—unidentified; 13—valine; 14—methionine and leucine; 15—threonine; 16—unidentified trace.

occasionally obtained on p-cresol, phenol, tyrosine, or guaiacol substrates whereas catechol, adrenaline, and, to an even greater extent, pyrogallol provided relatively excellent substrates for the system. From these data it appears that the enzyme which oxidizes the polyhydric phenols in snail liver is not identical with that reported for certain plant tissues (Nelson and Dawson, 1944). The enzyme described by these earlier workers (tyrosinase) was active against both polyhydric phenols and monohydric phenols, although the activity against the latter was usually somewhat unstable. It would seem that the enzyme described herein more closely approximates the catecholase studied by Graubard (1939) which also attacks only polyphenols. However, in this regard it should be remarked that the activity of tyrosinase against catechol has been reported as being *ca.* 124 times that against tyrosine (Kubowitz, 1938). Therefore, it was considered possible that the amount of tissue used in the major part of the present study was insufficient to demonstrate the action against tyrosine and other monophenols, especially since the maximum polyphenol oxidase activity measured was considerably less than that demonstrable on potato and mushroom preparations. In testing this, however, no combination of reactants was found which at any time permitted anything but periodic and highly insignificant activity with monophenol substrates.

TABLE IV

Survey of polyphenol oxidase (catecholase) activity in snail tissues. Reaction mixture as in Table III

Tissue	Q_{O_2}		
	No. 1	No. 2	No. 3
Liver	105	108	107
Foot	29	22	20
Mantle	68	64	55

In view of the demonstrated inactivity of snail tissues against monophenols, liver extracts were qualitatively analyzed for free amino acids, using two-dimensional ascending paper partition chromatography with phenol and 2, 4-lutidine as solvents, to determine the presence and metabolic availability of tyrosine as a substrate for enzyme activity *in vivo.* Glutamic acid, aspartic acid, valine, β-alanine, serine, glycine, arginine, lysine, alanine, proline, leucine, methionine, citrulline and/or glutamine were readily identifiable in good quantity but at no time was there detected any appreciable amount of tyrosine (Fig. 1). This apparent absence of tyrosine is consistent with the failure of the tissue preparations to oxidize monohydric phenols and the failure of the intact snail to produce pigment from tyrosine substrates.

Although maintaining the snails in the presence of catechol for 7–14 days prior to the enzyme assays failed to elicit any increased activity, it was observed that a decline in enzyme activity accompanied the decolorization of the tissues of snails kept for several weeks in tap water without food. This observation seems to associate the enzyme action with the pigmentation of the snail *in vivo,* particularly since other metabolic systems not concerned with melanin production (respiratory enzymes, transaminase, and the amino acid oxidases) were not similarly affected (current unpublished results).

Further evidence of an association of the polyphenol oxidase activity with pigmentation was given as the result of enzyme studies on other tissues of the snail (Table IV). Both the mantle and the foot, in order, showed some activity but far less than that described above for the liver. It was interesting to note that the level of activity in the three tissues corresponded roughly to the degree of pigmentation, the foot in this snail being relatively free of coloration.

Acknowledgment is made to Dr. Harold Harry, U. S. Army Tropical Research Medical Laboratory, San Juan, Puerto Rico, for the initial collecting of the snails used in this study.

Summary

A study was made on the polyphenol oxidase activity of snail tissues. The liver showed higher activity than either the mantle or foot; however, even in this organ only polyhydric phenols were effectively oxidized. No consistent activity on monophenol substrates was found. The possible association of the polyphenol oxidase system to the heavy pigmentations in the liver was discussed.

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AN ANALYSIS OF CELL GROWTH AND METABOLISM IN THE CRAYFISH (PROCAMBARUS ALLENI)

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In a recent paper I proposed a general scheme relating cell growth to organism growth with special reference to the problem of body size and metabolic rate (Davison, 1955). Three hypothetical cases were considered. (1) If growth of the animal is exclusively by cell enlargement, cell surface and external body surface each vary directly as the body weight raised to the 2/3 power. (2) If growth is characterized by the progressive formation of cells of fixed size, cell surface will be directly proportional to the body weight raised to the one power. (3) Growth involving the simultaneous addition and enlargement of cells results in cell surface varying as the body weight raised to some power intermediate between 2/3 and 1. In the 1955 paper it was demonstrated that post-metamorphosis growth of frog skeletal muscle is strictly by enlargement of fibers present at the completion of metamorphosis, no new fibers being formed. Both metabolic rate (cc. O_2/hr./gm.) and muscle surface concentration were directly proportional to the body weight raised to the $-1/3$ power.[1] Thus the metabolic rate of the whole animal was directly proportional to the surface concentration of the skeletal muscle cells.

In the frogs studied, the geometric weight increases were small (10- to 50-fold). In further pursuing the problem, I thought it might prove interesting to examine an animal which undergoes a large geometric increase body weight. The crayfish (*Procambarus alleni*) weighs about 4 mg. at the time it becomes independent of the parent crayfish, and increases to an average adult weight of about 16 gm., a 4000-fold increase in body weight. If such growth were isogonic, and the result of cell enlargement, cells would undergo a 13-fold increase in linear dimension ($4000^{\frac{1}{3}}$). Similarly, if the surface law of metabolism were in effect, metabolic rate would undergo a 13-fold decrease over this weight range. Since such dramatic changes in metabolism and cell dimensions seemed unlikely, the crayfish was selected as an interesting object for investigation.

MATERIALS AND METHODS

Animals

Here in the zoology department a breeding population of *P. alleni* has maintained itself in a concrete pool for about 5 years. The pool has a surface area of

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[1] The usual form of the surface law equation is:

$$\text{cc. } O_2/\text{hr.} = k\ W^{\frac{2}{3}}$$

It is more convenient to express metabolism in the form of intensity, thus making it comparable with other information obtained in the form of concentration. Dividing both sides by grams:

$$\text{cc. } O_2/\text{hr./gm.} = k\ W^{-\frac{1}{3}}$$

Similarly, cm.^2 surface $= k\ W^{\frac{2}{3}}$ becomes:

$$\text{cm.}^2\ \text{surface/cm.}^3\ (\text{gms.}) = k\ W^{-\frac{1}{3}}$$

about 3 m.^{2} and is about 10 cm. in depth. Weak daylight is augmented by continuous illumination from a 300-watt bulb. In addition to the algae which grow in the pool, food is provided in the form of occasional frog carcasses and pellets of mouse food. The population usually consists of 10 to 15 adult animals with a variable number of young depending on the breeding history of the pool. The population can be assumed to be genetically quite homogeneous, since often the surviving adults are siblings from a single successful breeding.

Determination of respiratory rate

P. alleni is of the chimney-building variety and is able to survive under moist terrestrial circumstances. Adult animals frequently leave the pond and migrate about the laboratory, especially during rainy weather. It is thus possible to determine the respiratory rate in air with simple volumetric instruments similar to that employed by Flemister and Flemister (1951). These consist simply of two chambers separated by a manometer, one vessel containing the animal and CO_2 absorbent, and the other serving as a compensating vessel. The total volume of the animal chamber can be varied by means of a calibrated syringe, and the gas consumed is measured as the decrease in volume of the animal chamber required to maintain equal pressure in the two vessels. Three volumetric instruments were used. The first two, for larger animals, utilized animal chamber volumes of *ca.* 500 and 50 cc. and oxygen uptakes were measured with 10- and 1-cc. syringes, respectively. The third instrument consisted of two 5-cc. vials connected by a manometer. The volume of the animal chamber was varied by means of a mercury column in a 0.2-cc. calibrated pipette, driven by an ordinary screw clamp. For the smallest animals (4 to 35 mg.) simple capillary respirometers were employed, consisting of vertically mounted capillaries (*ca.* 0.75 mm. bore) attached to horizontal vessels by means of No. 00 rubber stoppers. Vessel volumes were about 0.2 cc. for the smaller and 0.5 cc. for the larger animals. Four instruments, closely matched for final gas volumes and capillary bores, were used in a determination, three being experimental vessels with the fourth serving as a control and thermobarometer. A three-mm. column of kerosene was used as the indicator drop. Results obtained with the capillary instruments agreed well with those obtained with the volumetric instruments on animals of comparable weight. All instruments were carefully calibrated with mercury.

Filter paper, saturated with 10% KOH, was used as CO_2 absorbent, with plastic screening protecting the animal from injury. Experiments in which pure oxygen was flushed through the instrument did not differ significantly from experiments in air. Accordingly, the experiments reported here were performed in atmospheric air. Heistand (1931) reports a T_c of about 40 mm. Hg for oxygen consumption in *Cambarus,* a value well below that approached in the present experiments. The water bath was maintained at 25.1 ± 0.05 C.°

Twenty-four to 48 hours prior to a determination, the animals were isolated in finger bowls or glass aquaria and maintained without food in order to minimize digestive and absorptive activity. After introduction into the respirometer, oxygen consumption was followed with the animal at rest until uptake became linear with respect to time. After the preliminary equilibration of 15 to 30 minutes, most determinations lasted from 60 to 180 minutes. Only one animal was used per respirometer. The animals were blotted with filter paper and weighed fresh following the determination. No sex differences were observed. Oxygen consumption is expressed as cc. O_2/hr./gm. fresh weight, and gas volumes are reduced to standard temperature and pressure (760 mm. Hg and 0° C.).

No seasonal influence on breeding is apparent in the population, and the metabolism measurements reported here were obtained between September and March.

Determination of cell surface

The animal was weighed, decapitated and the abdomen removed. A small portion of abdominal flexor muscle was dissected out and teased apart on a glass slide. The teased muscle was then stained with 0.75% acetocarmine in 45% acetic acid. It is necessary to stain the muscle cell nuclei in order to easily identify single fibers. The preparation was observed under a suitable power of the compound microscope. About 20 fibers were chosen at random, and the fiber diameters projected on paper by means of the camera lucida. A known linear dimension was projected from a stage micrometer, and the mean fiber diameter determined. For a sample of 20 fibers, the standard deviation was found to be about 20% of the mean. Assuming the fibers approximate long cylinders without free ends, their

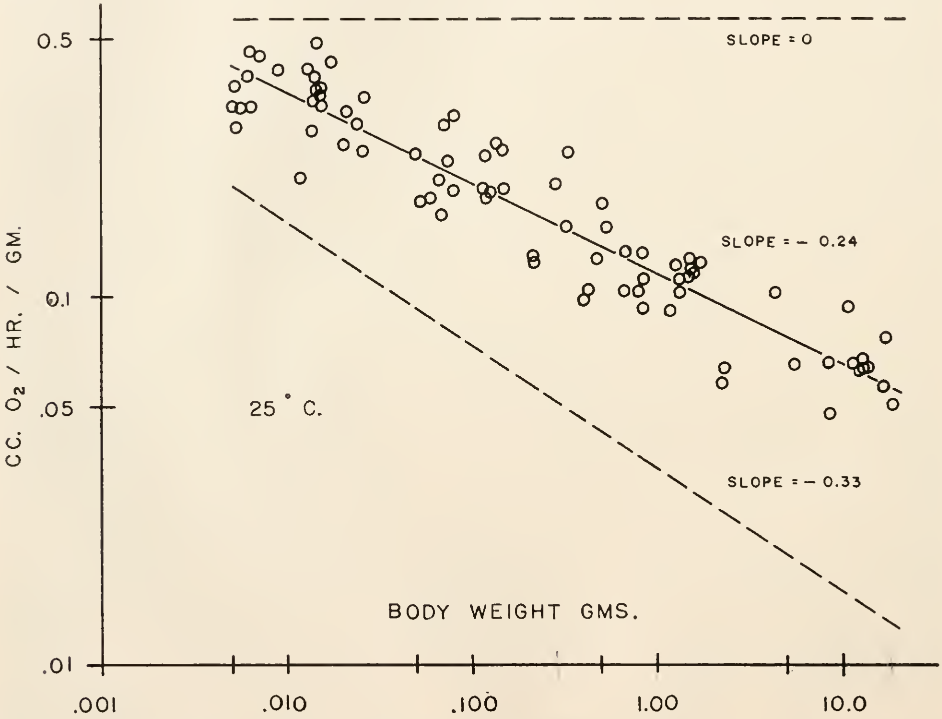

FIGURE 1. Resting oxygen consumption in *Procambarus alleni* plotted against the body weight in grams. Both ordinate and abscissa are in log form. The equation of the experimentally determined line is: cc. O_2/hr./gm. $= 0.115\ (W)^{-0.24}$. 25° C.

surface is given by $2\pi rl$ and their volume by $\pi r^2 l$, so surface per unit volume is the former over the latter or 2/r (4/d). This quantity, expressed as cm.^{2} surface/cm.^{3} fibers, is defined as the surface concentration of the muscle. Examination of fiber cross-sections indicates extra-fibral spaces less than 5% of the total area of the section.

Other measurements

Measurements were also made of abdomen length and breadth, eye diameter, and ommatidia facet size. The decapod ommatidium is a receptor unit of a definite number of cells (Schneider, 1902), so that an analysis of eye size and facet size allows an indirect examination of cell growth and organ growth using the familiar allometric equation of Huxley (1932). Eye diameter was obtained by observing the animal in dorsal aspect and taking[2] the diameter parallel to the longitudinal axis of the animal. Abdomen length was taken as the dorsal distance between the posterior margin of the cephalothorax and the base of the telson. Abdomen breadth is the ventral distance between the pleura of the first abdominal segment.

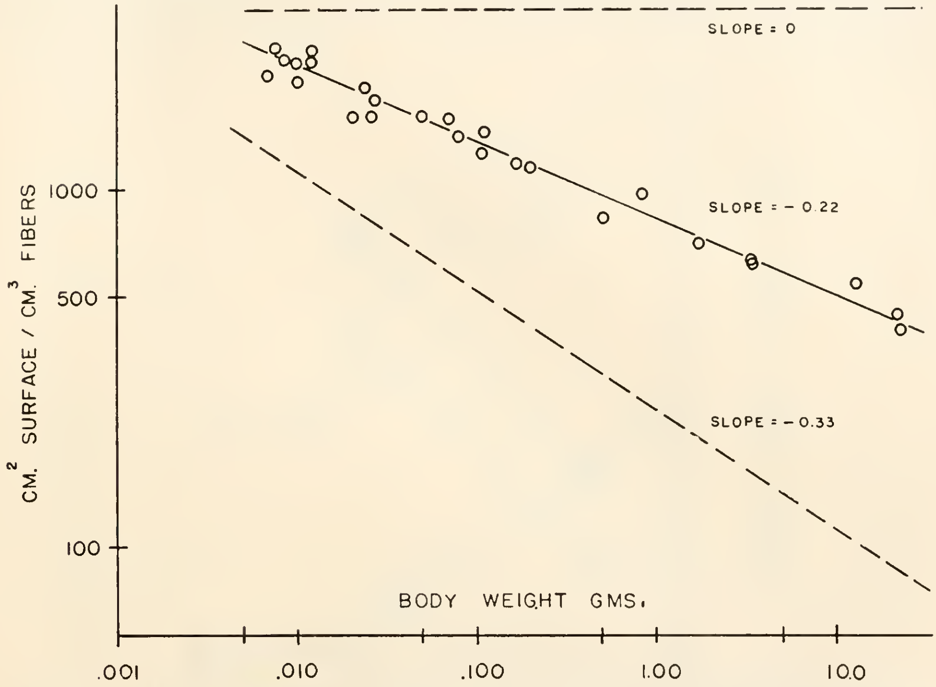

FIGURE 2. Muscle surface concentration plotted against body weight in grams. Both ordinate and abscissa are in log form. The equation of the experimentally determined line is: cm.^{2} surface/cm.^{3} fibers = 830 $(W)^{-0.22}$.

The size of the corneal facets, which are square in the crayfish, was determined by tracing the outline of a suitable number (20–25) from the most distal part of the eye, again using camera lucida. The most distal facets are about three times as long on a side as those near the base of the eye. The average length on a side of the distal facets was recorded together with the data on eye diameter, abdomen length and breadth, and the body weight in grams.

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[2] $y = b x^k$. y = linear dimension of organ or cell; x = linear dimension of reference organ or the comparable dimension derived from the body weight ($W^{\frac{1}{3}}$); b = constant of proportionality; k = allometric coefficient, indicated by the slope when log y is plotted versus log x.

RESULTS

Respiratory rate

In Figure 1 is plotted resting metabolic rate versus body weight in grams, each scale being in logarithmic form. The straight line, drawn by eye, has a slope of −0.24, a value intermediate between 0 (metabolic rate independent of body weight) and −0.33 (metabolic rate following the surface law). Metabolic rate decreases from about 0.355 cc./hr./gm. at 10 mg. to 0.066 cc./hr./gm. at 10 gm., about a 5.4-fold decrease in rate, and considerably less than the 10-fold decrease expected on the basis of the surface law. Weymouth *et al.* (1944) reported a similar value for the exponent (−0.20) in a study of the metabolism of *Pugettia* and other marine crustacea. In *Procambarus,* the rate at 10 gm. (0.066 cc./hr./gm.) compares favorably with the respiratory rate reported for the European lake crayfish *Astacus leptodactylus* (0.07 cc./hr./gm. at 20 C.°) (Prosser *et al.,* 1950).

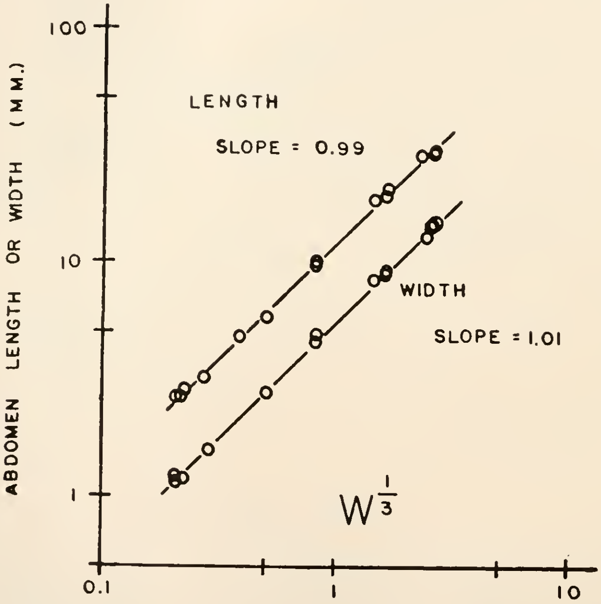

FIGURE 3. Abdomen length (upper plot) and abdomen width (lower plot) plotted against the cube root of the body weight in grams. Both coordinates are in log form. The equations of the lines are as follows: Abd. length (mm.) $= 12\ (W^{\frac{1}{3}})^{0.99}$. Abd. width (mm.) $= 5.5\ (W^{\frac{1}{3}})^{1.01}$.

Muscle cell surface

Figure 2 represents the calculated surface concentrations obtained from average fiber diameters plotted against body weight in grams, again in double logarithmic form. The straight line, fitted by eye, indicates a slope of −0.22, indicating that growth of the muscle is accomplished by the enlargement of fibers, once formed, and the progressive addition of fibers throughout the weight range. By exploring the teased and stained preparations, it is possible to observe stages in fiber formation. Spindle-shaped myoblasts come to lie together in closely packed longitudinal groups, with subsequent loss of their cell boundaries, and a peripheral arrangement of the fiber nuclei. (For a discussion of the histogenesis of decapod skeletal muscle see Schneider, 1902.) It is not clear from the present study at exactly which stage in fiber growth the myoblasts stop contributing to the fiber. However, the spacing of the nuclei is much greater in large than in small fibers, indicating that the individual fiber receives all its nuclei while still relatively small.

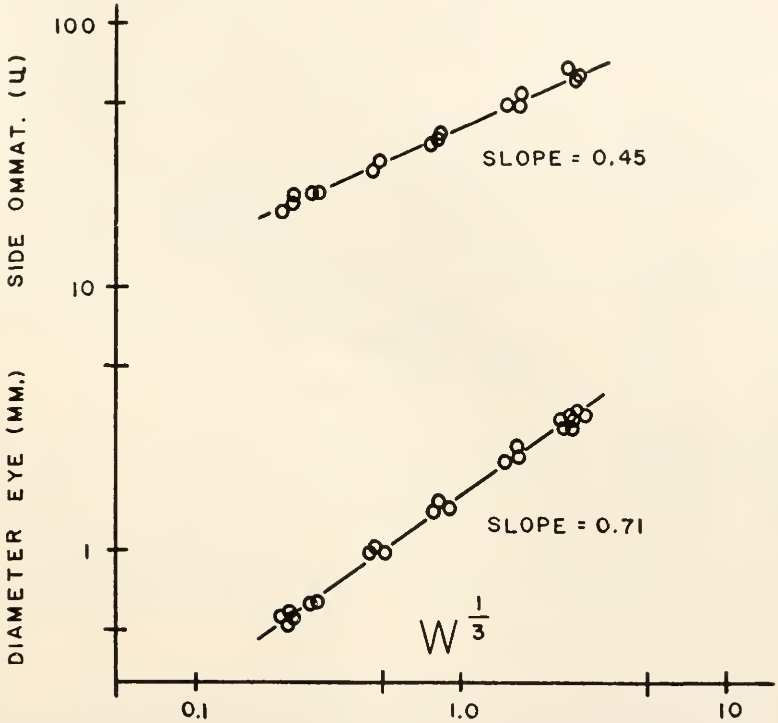

FIGURE 4. Eye diameter and ommatidia facet length plotted against the cube root of the body weight in grams. Both coordinates are in log form. The equations of the lines are as follows: side ommatidium (μ) $= 39.8\ (W^{\frac{1}{3}})^{0.45}$, diameter eye (mm.) $= 1.62\ (W^{\frac{1}{3}})^{0.71}$.

Surface concentrations decrease from about 2240 cm.^{2}/cm.^{3} at 10 mg. to 500 cm.^{2}/cm.^{3} at 10 gm., corresponding to about a 4.5-fold increase in fiber diameters. The lower dotted line in Figure 2 indicates the 10-fold decrease which would occur if the fibers grew entirely by enlargement.

The observed 4.5-fold increase in fiber dimensions allows an indirect calculation of the increase in fiber numbers for a 1000-fold increase in weight. It may be assumed that fiber lengths are determined by the anatomical features of the origins and insertions, and accordingly undergo a 10-fold increase between 10 mg. and 10 gm. Since fiber diameters would also increase by a factor of 10 if their number remained constant, the relative increase in number of fibers is given by $(10/4.5)^2$ or 4.9. Thus, a weight increase of 1000-fold is accompanied by about a 5-fold increase in fiber number.

An alternative possibility to the addition of fibers during growth is that the abdomen might be markedly heterogonic with respect to the body as a whole. In order to test this possibility, abdomen length and breadth have been plotted against the dimensionally comparable quantity derived from the body weight (cube root of weight). The double log plot in Figure 3 indicates slopes for both length and breadth very close to unity, being 0.99 and 1.01, respectively. In other words, the relationship between fiber dimensions and body size is essentially identical to that between fiber dimensions and abdomen size. The abdomen and the whole body are isogonic.

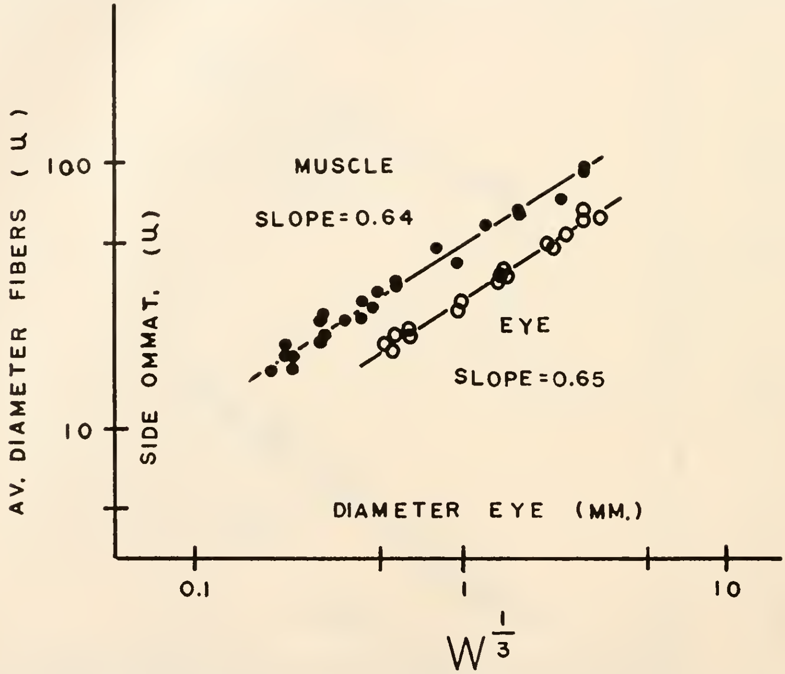

FIGURE 5. Average diameter muscle fibers plotted against the cube root of the body weight in grams (upper plot), and ommatidia facet length plotted against the diameter of the eye (lower plot). All coordinates are in log form. The equations of the lines are as follows: Av. fiber diam. $(\mu) = 49\ (W^{\frac{1}{3}})^{0.64}$; side ommatidium $(\mu) = 29.5\ (\text{diam. eye (mm.)})^{0.65}$.

Ommatidia and eye growth

The eye and its ommatidia are especially interesting in this connection, since each is markedly heterogonic with respect to body weight. Figure 4 illustrates the allometric relationship between eye diameter (mm.) and ommatidia facet length (μ) when each is plotted against $W^{\frac{1}{3}}$. The slopes for each are less than unity, 0.71 for the eye, and 0.45 for facet length. The lower value for facet length indicates that growth of the eye is characterized by the progressive addition of receptor units. Knowing the allometric coefficient (slope) for each with respect to $W^{\frac{1}{3}}$, it is possible to calculate the allometric coefficient of ommatidia size with respect to eye size. This will be the ratio of the slopes or 0.45/0.71 which equals 0.635.[3]

Since average muscle fiber diameters increase systematically with body weight, it should also be possible to express this growth according to Huxley's allometric formulation. In Figure 5 are plotted both average fiber diameter (μ) against W (upper plot) and, on the basis of direct measurements, facet length (μ) against eye diameter (mm.). Fiber diameter indicates a slope of 0.64 and facet length a slope of 0.65, each value agreeing closely with the calculated slope (0.635) for facet length versus eye diameter obtained above.

Since the eye is a two-dimensional (surface) system of receptor units, the relationship between number of ommatidia and size of the eye will be essentially identical with that between number of fibers and size of the abdomen, in which the increase in number is also dependent on two-dimensional quantities (cross-sectional area of the abdomen and the fibers).

The close agreement of the allometric coefficients for the ommatidia-eye system and the fiber-body system indicates that the same basic mechanism of simultaneous addition and enlargement operates in each.

DISCUSSION

It is clear, then, that a departure from the surface law of metabolism has been associated with a quantitatively similar departure in the manner of cell growth. Both metabolic rate and muscle surface concentration vary as the body weight raised to a power intermediate between − 0.33 and 0. These observations are consistent with the view that the metabolic rate of the whole animal may be determined by the cell surface of the skeletal muscle. In addition, another organ system, the eye, indicates a manner of growth which is quantitatively comparable to that of the body as a whole.

Huxley (1932) has compiled a list of arthropod allometric coefficients drawn largely from Teissier's paper (Teissier, 1931). In the European walking stick (*Dixippus morosus*) values of the allometric coefficient are 0.48 and 0.37 for the eye and ommatidia diameters, respectively (measured against total length), giving a value of 0.77 (0.37/0.48) for ommatidia diameter versus eye diameter, and indicating a manner of growth comparable to that observed in the present study. Trager's analysis indicates that the tissues of the flesh fly larva (*Lucilia sericata*) increase in size solely as the result of cell enlargement (Trager, 1935). He also cites a number of earlier papers suggesting the growth of larval tissues by cell enlargement. Among these is a study by August Weismann (1864) who noted an increase in size but not in number of muscle cell nuclei during the larval growth of muscid flies.

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[3] $\log\ (\text{facet length}) = 0.45 \log\ (W^{\frac{1}{3}}) + \log b$, and: $\log\ (\text{eye diameter}) = 0.71 \log\ (W^{\frac{1}{3}}) + \log b'$. Solving each for $\log\ (W^{\frac{1}{3}})$: $\frac{\log\ (\text{facet length})}{0.45} - \log b = \log\ (W^{\frac{1}{3}}) = \frac{\log\ (\text{eye diameter})}{0.71} - \log b'$, and eliminating the common term: $\log\ (\text{facet length}) = 0.45/0.71 \log\ (\text{eye diameter}) + \log b - \log b'$.

I would like to point out that the findings of the present study, as well as those cited by reference, are not in agreement wth Zeuthen's repeated statements to the effect that metazoan growth is characterized by an increase in number and not in size of cells (Zeuthen, 1953, 1955).

However, Zeuthen has made what may be a very pertinent observation when considered from the point of view developed here. Considering the animal kingdom as a whole, he points out that both in very small (less than a few mg.) and in very large (several kg.) animals, metabolic rate tends to change less markedly with body weight than in animals from the middle range of weight (Zeuthen, 1949). A possible explanation for this phenomenon may reside in a consideration of cellular structure. In very small animals a practical lower limit in cell dimensions may be reached so that smaller animals are composed of fewer rather than smaller cells. Similarly, there may also exist an upper limit on cell dimensions beyond which larger animals would be composed of more rather than proportionately larger cells.

Bertalanffy and co-workers (1951, 1953) prefer to attribute the phenomenon of size-dependent metabolism to "organismic factors." Since the phenomenon is expressed by the intact organism, there seems little doubt that organismic factors will play their part. However, cellular structure is not thereby precluded as a determining or, under standard conditions, a limiting factor in the expression of metabolism by the whole animal. The argument developed here points to a view of the organism as a highly integrated cell aggregate or collection of unit organisms (cells) whose growth, and hence structural, properties determine the performance of the animal proper. Virchow expressed a similar, if oversimplified, view of the organism in 1858 when he said, "Every animal appears as a sum of vital units, each of which bears in itself the complete characteristics of life." [4]

SUMMARY

1. The resting metabolism of *Procambarus alleni* was determined over about a 4000-fold weight range. Metabolic rate was found to vary directly as the body weight raised to the -0.24 power.

2. Abdominal muscle fiber diameters were determined over about a 4000-fold weight range. Calculated surface concentration was found to vary directly as the body weight raised to the -0.22 power, providing evidence that the growth of the skeletal muscle is characterized by simultaneous addition and enlargement of fibers throughout the weight range.

3. Allometric analyses were made of fiber diameter, eye diameter, and ommatidia size with respect to the cube root of the body weight. The analyses indicated that the growth of the eye with respect to its constituent units is quantitatively similar to the growth of the abdomen with respect to its skeletal muscle cells.

4. The close similarity between the allometric coefficients of metabolic rate and surface concentration was taken as evidence that the metabolic rate of the whole animal may be dependent on the cell surface of the skeletal muscle.

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[4] Quoted in introductory chapter to E. B. Wilson, The cell in development and heredity, 1925.

5. Evidence obtained from the literature was cited for other examples of the growth of arthropod tissues by cell enlargement.

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PHASE DIFFERENCE IN THE TIDAL RHYTHMS OF COLOR CHANGE OF TWO SPECIES OF FIDDLER CRAB[1]

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Persistent behavior patterns correlated with cosmic events have been reported for numerous animals. Brown, Fingerman, Sandeen and Webb (1953) recently reviewed the extensive literature dealing with persistent tidal rhythms in invertebrates. Bohn (1903) and Gamble and Keeble (1903, 1904) were the first investigators to report a rhythm with tidal frequency. A flatworm, *Convoluta roscoffensis,* was observed to emerge onto the surface of the sand at low tide and disappear into the sand as the tide rose. This rhythmic activity persisted in laboratory aquaria in the absence of contact with the tides.

Pigment in the melanophores of fiddler crabs, *Uca,* exhibits a daily rhythm of migration causing the animal to be dark by day and light by night. This daily rhythm persisted for at least several weeks in constant darkness in the laboratory without loss of synchrony with solar day-night. The frequency of this rhythm is independent of temperature between 6° and 26° C. (Brown and Webb, 1948).

Brown, Fingerman, Sandeen and Webb (1953) demonstrated a persistent tidal rhythm of migration of the pigment in the melanophores of *Uca pugnax.* The tidal rhythm of *Uca pugnax* expressed itself by a supplementary dispersion of the pigment in the melanophores one to three hours later than low tide. A low tide occurs twice every lunar day on the beaches near Woods Hole, Massachusetts, where the *Uca pugnax* were collected. The tidal rhythm is superimposed upon the daily rhythm and leads to a third type, a semilunar rhythm, because the daily and tidal rhythms are in the same phases with respect to one another only once every 14.8 days.

The phases of the tidal rhythm bore a definite relationship to the times of low tide in the native habitat of the *Uca pugnax.* Fiddler crabs collected from two diverse localities where the times of low tide differed by four hours showed in the laboratory persistent tidal rhythms in phase with the tidal changes of their respective original habitats. The tidal rhythms of the two groups of *Uca* were out of phase with each other by approximately four hours.

The frequency of the tidal rhythm of pigment migration in the melanophores of *Uca pugnax* is independent of temperature between 13° and 30° C. (Brown, Webb, Bennett and Sandeen, 1954). Hines (1954) demonstrated a tidal rhythm in the behavior of the melanophores in autotomized legs of *Uca pugnax.*

Fingerman (1955) found a tidal rhythm of migration of the pigment in the melanophores of the blue crab, *Callinectes sapidus.* The form of this tidal rhythm was similar to the form of *Uca pugnax*. The *Callinectes* were collected in the vicinity of New Orleans, Louisiana, where the tides are chiefly diurnal. The tidal rhythm of *Callinectes* expressed itself through a supplementary dispersion of the melanin in the chromatophores about one hour after both the low and high tides, which occur 12.4 hours apart in a region with diurnal tides.

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[1] This investigation was supported by Grant No. B-838 from the National Institutes of Health.

The current investigation was initiated in order to determine the character of the tidal rhythm of two species of *Uca* from a region of diurnal tides and to compare the rhythms of these *Uca* with the rhythm of the *Uca pugnax* which were collected in a semi-diurnal tidal habitat.

Materials and Methods

Adult male and female specimens of *Uca pugilator* and *Uca speciosa* were collected on the beach near the Gulf Coast Research Laboratory at Ocean Springs, Mississippi, and transported to an air-conditioned laboratory in New Orleans, Louisiana, for use in the observations to be described. From the evening of the day of collection until the end of the period of observation the crabs were maintained in darkness except for the few minutes required to make the observations. The temperature in the laboratory was 20° C. on the day the first *Uca* were collected. The temperature increased gradually throughout the spring and summer. On the day the last observations were performed, the temperature in the laboratory was 24° C. The *Uca* were kept in stainless steel aquaria containing sea water. The aquaria were inclined so that the bottom was not completely covered with sea water. The *Uca* were able to move into and out of the water. The sea water in the aquaria was changed every second day at 7:15 P.M.

The method of staging the chromatophores was the same as described by Brown, Fingerman, Sandeen and Webb (1953). The average chromatophore index of the pigment in the melanophores on the anterior aspect of the walking legs was determined with the aid of a stereoscopic dissecting microscope and microscope lamp hourly from 8 A.M. through 7 P.M. every second day. The chromatophore index of Hogben and Slome (1931) was employed to stage the melanophores. The most concentrated state of the pigment is described as stage 1, the most dispersed as stage 5, and the intermediate conditions as stages 2, 3, and 4.

Table I lists the times of high and low tide at Ocean Springs on the days observations were performed in the laboratory. Because of the astronomical conditions during this portion of the year the tides did not follow a consistent pattern and were not regularly spaced 12.4 hours apart. The times were obtained from the tide tables published by the Coast and Geodetic Survey, United States Department of Commerce.

Results

The tidal rhythm of Uca pugilator

On June 1, 1955, approximately 2000 *Uca pugilator* were collected. The crabs were divided into two groups and placed in darkness in the laboratory at 7 P.M. The animals from this collection will be referred to as the *Uca* of Series A. For the hourly determinations of the chromatophore index, 50 *Uca* were taken alternately from each lot. Series A was observed every second day from June 2 through

July 20, 1955. The hourly chromatophore indices obtained are presented in Table II.

The data obtained from the Series A *Uca* on June 12 through June 30 were used to prepare Figure 1. These data were selected because they illustrate the nature of the tidal rhythm. On June 12 (Fig. 1A) the peak of the curve was far to the right. Two days later on June 14 (Fig. 1B) the peak had shifted to the left. The peak then gradually shifted to the right again (Fig. 1C, D, E, F, and G). In Figure 1H (June 26) the peak was far to the right as in Figure 1A. As seen in Figure 1I (June 28) the peak had again shifted to the left just as in Figure 1B. The peak shifted day by day at the same rate as the tides. The shift of the peak from the right to the left is due to the tidal influence making its appearance early in the morning. The tidal effect became progressively later in the evening until it had no ability to maintain a peak late in the day. The tidal rhythm was superimposed upon the daily dispersion of the pigment in the melanophores as has been described for *Callinectes sapidus* and *Uca pugnax*.

TABLE I

The times of low and high tide at Ocean Springs, Mississippi, on days observations were made

	High tide	Low tide
June 2	7:52 A.M.	7:11 P.M.
4	8:58	8:25
6	10:12	9:44
8	11:31	10:51
10	12:46	11:34
12	1:36 P.M.	11:27
14	6:46 A.M.	5:23
16	6:58	6:09
18	8:18	7:50
20	10:02	9:38
22	11:51	11:03
24	1:22 P.M.	11:20
26	6:14 A.M.	8:36
28	5:42	5:33
30	6:43	6:30
July 2	8:02	7:46
4	9:28	8:53
6	10:48	9:46
8	12:01	10:13
10	1:03 P.M.	9:46
12	4:59 A.M.	4:30
14	5:40	5:14
16	7:15	6:57
18	9:13	8:38
20	11:08	9:59

Figures 1H and 1I were obtained 14 days after Figures 1A and 1B, respectively. The 14 days required for the passage of the rhythm is the approximate duration of a semilunar rhythm.

Calculation of the exact relationship between the times of high and low tide and the phases of the tidal rhythm were impossible because of the peculiarity of the tides at this time of the year. The general relationship between the time of the tide and the time of maximal tidal effect upon the melanophores previously observed with *Uca pugnax* and *Callinectes sapidus* holds in the results reported here. The maximal tidal effect occurred about two hours after the time of the tide.

To illustrate better the nature of this tidal rhythm Figure 2 and Table III have been prepared in the following manner from the data presented in Table II. The 12 periods of observation were divided into four periods of three hours each, 8–10 A.M., 11 A.M.–1 P.M., 2–4 P.M., and 5–7 P.M. The percentage of the total daily excursion that each three-hour period occupies was then calculated. The 12 hourly averages were summed and 12 subtracted from the total because if there had been no daily excursion of the pigment the sum would have been 12. Similarly, the average chromatophore indices for each three-hour period were summed and three subtracted from the total. The percentage of the total that each three-hour period occupied was then calculated. In Figure 2 the circles are the values calculated from the data of the *Uca pugilator* of Series A. The tidal maxima and minima progressed across the scale at a rate closely approximating the rate at which a tidal rhythm would be expected to progress. The parallel diagonal lines have been drawn through the tidal maxima at a distance of 14.8 days apart. Fourteen and eight-tenths days is the expected interval between days on which a low and a high

TABLE II

The average melanophore index for each of the 12 daily observations of the Uca pugilator of Series A

	8 A.M.	9	10	11	12	1 P.M.	2	3	4	5	6	7
June 2	2.7	3.5	2.9	3.3	2.5	3.0	3.0	3.0	2.2	3.0	2.5	2.6
4	2.9	3.1	2.8	3.7	2.9	3.3	2.8	3.0	2.6	2.6	2.2	2.6
6	3.0	3.1	3.0	3.3	3.1	3.7	3.1	3.6	3.1	3.1	3.3	2.8
8	2.8	3.0	3.3	3.1	3.5	4.1	3.9	4.0	3.1	3.5	3.1	2.8
10	3.6	3.3	3.2	3.7	3.6	3.8	3.3	4.3	3.8	4.0	3.5	3.5
12	3.5	3.5	3.4	3.5	3.4	3.6	3.6	3.8	3.7	3.9	3.5	3.5
14	3.7	4.7	3.9	4.1	3.1	3.7	3.4	3.5	3.3	4.0	3.4	3.6
16	4.0	3.6	3.6	3.8	3.4	3.5	2.8	3.2	2.9	3.1	2.6	2.7
18	3.5	3.6	3.4	3.9	3.8	3.5	3.3	3.5	3.2	3.0	2.8	2.5
20	3.3	3.9	3.6	3.2	3.2	3.8	3.2	3.5	3.5	2.9	2.4	2.5
22	3.2	3.4	3.2	3.5	3.2	3.4	3.6	3.2	3.5	3.6	2.6	3.1
24	3.0	2.9	3.1	3.2	3.4	3.0	3.3	3.4	3.8	3.3	3.3	2.6
26	3.1	3.3	2.8	3.0	3.1	3.3	3.2	3.2	3.3	2.9	3.3	3.3
28	3.1	3.2	3.3	2.9	2.7	2.5	2.9	2.4	2.8	2.7	3.1	2.5
30	3.2	2.7	3.2	2.6	2.6	2.3	2.4	2.4	2.4	2.5	2.1	2.1
July 2	2.8	3.0	2.9	2.2	2.5	2.4	2.5	2.0	2.6	2.2	2.4	2.2
4	2.9	3.0	2.7	2.8	3.2	3.1	3.2	2.7	2.6	2.4	2.5	2.3
6	2.8	2.6	2.8	3.3	2.7	2.7	2.9	3.0	2.8	3.1	2.2	2.6
8	2.7	2.4	2.8	3.5	2.5	3.0	2.8	3.2	2.7	3.0	2.2	2.4
10	2 6	3.1	2.7	2.9	2.6	3.3	2.9	2.9	2.8	2.8	2.8	2.5
12	2.5	2.5	2.5	2.5	2.5	2.6	2.7	3.1	3.1	2.7	2.4	2.8
14	2.5	3.1	2.8	2.5	2.2	2.0	2.2	2.3	2.3	2.3	1.9	2.1
16	2.5	2.6	2.3	2.2	1.9	2.3	1.9	1.9	1.9	2.2	1.9	1.6
18	2.7	2.6	2.0	2.5	2.1	2.2	2.1	2.2	2.3	2.0	2.0	2.1
20	2.4	2.9	2.5	2.4	2.2	2.7	2.8	2.5	2.1	2.5	2.3	2.0

tide will occur at the same time of day at any given location where the tides are diurnal in nature.

Comparison of the tidal rhythms of Uca pugilator and Uca speciosa

On June 15, 1955, 1000 *Uca pugilator* (Series B) were collected. The *Uca* of Series B were handled and observed in the same manner as the *Uca* of Series A with the one exception that all the *Uca* of Series B were maintained in one aquarium. Fifty *Uca* were observed at each reading. The data obtained from the observations of Series B were used in the preparation of Tables IV and V and Figure 2. The dots in Figure 2 are the data taken from Table V. As is evident from Figure 2, the *Uca pugilator* of both Series A and B were rhythmically similar although the *Uca* of Series B had been collected two weeks after the crabs of Series A. The tidal rhythm of the *Uca* in the laboratory during the first two weeks of June (Series A) must have progressed at the same rate as the rhythm of the *Uca pugilator* which

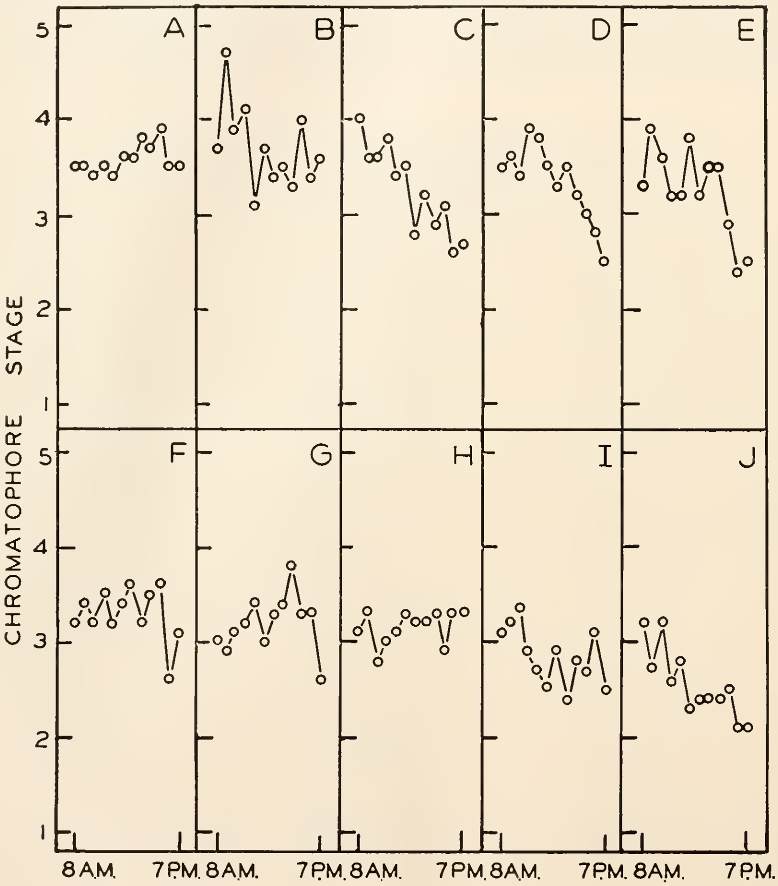

FIGURE 1. The daily pattern of the *Uca pugilator* of Series A observed every second day from June 12 (A) through June 30 (J).

were on the beach at Ocean Springs 93 miles to the east during the same period of time (Series B).

Seventy-one specimens of *Uca speciosa* (Series C) were also collected at Ocean Springs on June 15. The *Uca* of Series A, B, and C were treated in an identical manner. Fifty of the *Uca speciosa* were observed at each reading. When the number of animals had decreased to 50, all of the remaining *Uca speciosa* were observed. The number of individuals in Series C gradually decreased until 42 remained on the last day of observation (July 14). The data obtained with Series C were used to prepare Tables VI and VII. The *Uca pugilator* (Series A and B) were found to be six days out of phase with the *Uca speciosa* (Series C) as can be seen from Figure 3. In this figure sections A and B are the curves obtained from the *Uca pugilator* of Series A on June 26 and 28, respectively. Sections C and D represent the observations of the *Uca pugilator* of Series B on June 26 and 28. Sections E and F were taken from the observations of the *Uca speciosa* (Series C) for June 20 and 22, respectively. The curves for the *Uca pugilator* of both Series A and B were similar to one another as is evident from Figure 3. On June 26 the major peak of the curves from Series A and B was far to the right. On June 28 the major peak in Series A and B had shifted far to the left as the predominant tidal influence shifted from evening to early morning. A similar shift of peaks had oc-

TABLE III

The percentage of the daily melanin dispersion occurring at each of four periods of the day in Series A

	8–10 A.M.	11 A.M.–1 P.M.	2–4 P.M.	5–7 P.M.
June 2	27.5	26.1	23.4	23.0
4	25.8	30.7	24.0	19.6
6	23.3	27.1	26.0	23.7
8	21.6	27.3	28.4	22.7
10	22.5	25.6	26.6	25.4
12	24.0	24.3	26.2	25.6
14	28.7	24.4	22.2	24.7
16	30.1	28.3	21.7	19.9
18	26.8	29.3	25.0	18.9
20	28.9	26.7	26.7	17.8
22	24.7	25.8	26.5	23.0
24	22.8	25.1	28.5	23.6
26	24.0	24.8	26.0	25.2
28	29.9	23.1	23.1	24.0
30	32.6	25.2	22.5	19.8
July 2	32.2	23.2	23.2	21.5
4	26.2	28.5	25.7	19.6
6	24.2	26.5	26.5	22.8
8	23.1	28.3	26.9	21.7
10	24.7	26.5	25.6	23.3
12	22.6	23.1	29.7	24.6
14	33.9	22.8	23.5	20.4
16	33.3	25.8	20.5	20.5
18	29.0	25.7	24.3	21.0
20	27.7	24.9	25.4	21.9

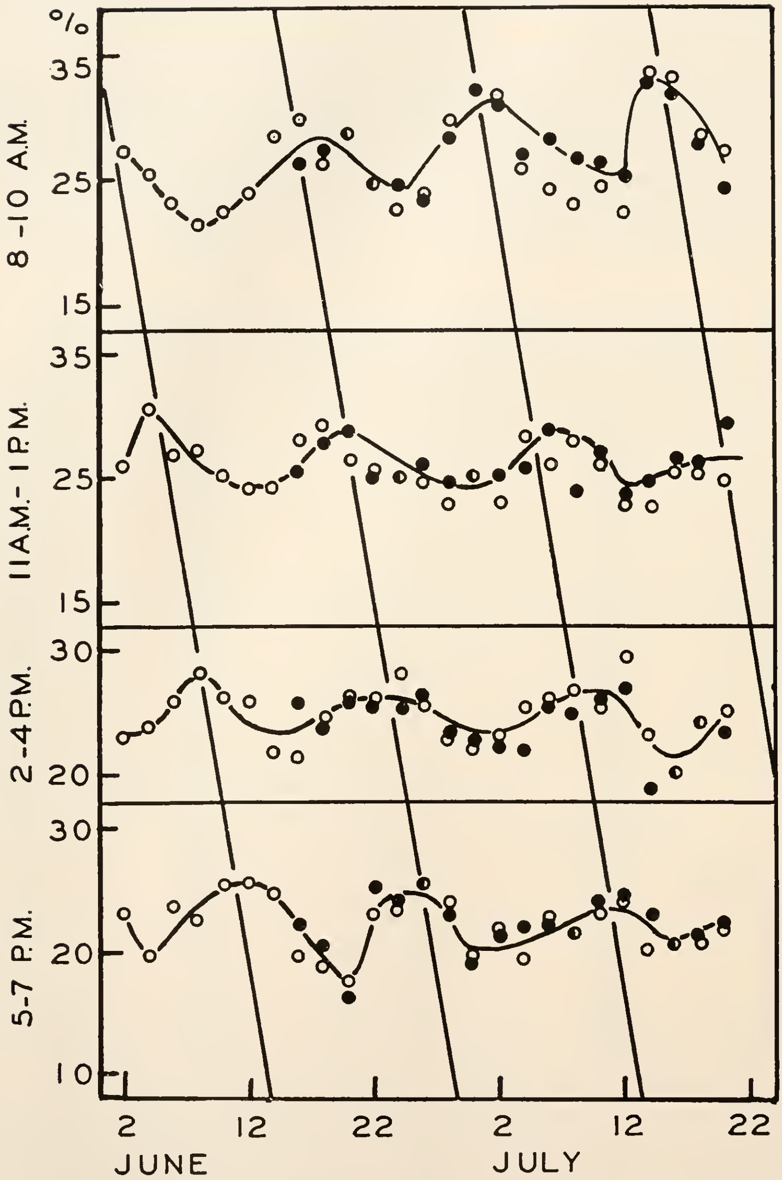

FIGURE 2. The relationship between the percentage of the daily melanin dispersion of *Uca pugilator* occurring at each of four periods during the day and the day of the month. Note the tidal maxima passing over the daily periods at the tidal rate of approximately 50 minutes per day. There is also a 14.8-day cycle, the interval between each of the parallel diagonal lines. The circles represent the *Uca pugilator* collected June 1 (Series A). The dots represent the *Uca pugilator* collected on June 15 (Series B).

TABLE IV

The average melanophore index for each of the 12 daily observations of Series B, Uca pugilator

	8 A.M.	9	10	11	12	1 P.M.	2	3	4	5	6	7
June 16	3.2	3.2	2.9	3.4	3.0	2.7	2.8	3.3	3.1	2.7	3.0	2.6
18	3.6	3.7	3.3	3.6	3.7	3.4	2.9	3.4	3.3	3.1	2.8	2.7
20	3.8	3.9	3.8	3.8	3.7	4.0	3.4	4.3	3.0	2.9	2.5	2.4
22	3.2	3.5	3.6	3.6	3.3	3.5	3.6	3.5	3.6	3.8	3.4	3.2
24	3.2	3.7	3.6	3.6	3.6	3.8	3.4	3.5	3.9	3.8	3.3	3.2
26	3.3	3.4	3.6	3.5	3.6	3.5	3.8	3.6	3.8	3.7	3.7	3.4
28	3.8	4.0	3.7	3.6	3.5	3.4	3.2	3.3	3.5	3.4	3.2	3.2
30	3.7	4.0	3.6	3.6	3.0	2.9	2.9	3.0	2.9	2.6	2.8	2.5
July 2	4.0	3.7	3.7	3.4	3.4	3.2	3.2	3.0	2.9	3.1	2.7	2.9
4	3.5	3.6	3.5	3.8	3.9	3.6	3.3	3.0	3.0	3.2	2.9	3.1
6	3.9	3.3	3.6	3.5	3.4	2.6	3.1	3.5	3.4	3.2	3.0	2.7
8	3.5	3.9	3.5	3.9	3.6	3.5	3.6	3.4	3.4	3.1	3.3	2.9
10	3.8	3.5	3.4	3.2	3.3	3.3	3.8	3.6	3.2	3.4	3.5	3.0
12	3.5	3.2	3.1	3.2	3.0	3.1	2.9	3.6	3.8	3.5	3.0	2.9
14	3.5	3.5	3.4	3.1	2.8	2.6	2.3	2.8	2.2	2.9	2.7	2.6
16	3.6	3.2	3.1	3.0	2.9	2.9	2.6	2.6	2.4	2.3	2.5	2.6
18	3.4	3.4	3.0	3.2	3.0	3.1	3.0	3.7	3.1	2.8	2.5	2.8
20	3.1	3.0	3.3	3.6	3.2	3.9	3.3	3.0	2.9	3.1	2.8	3.0

curred in the *Uca speciosa* of Series C six days prior to the shift in the animals of Series A and B.

Figure 4 was prepared in the same manner as Figure 2 to illustrate better the tidal phase difference between the two species. In Figure 4 the circles are the

TABLE V

The percentage of the daily melanin dispersion occurring at each of four periods of the day in Series B

	8–10 A.M.	11 A.M.–1 P.M.	2–4 P.M.	5–7 P.M.
June 16	26.4	25.5	25.9	22.2
18	27.6	28.0	24.0	20.4
20	28.8	28.8	26.1	16.3
22	24.5	24.8	25.8	24.8
24	24.5	26.1	25.5	23.9
26	23.6	24.6	26.5	25.2
28	28.5	25.2	23.5	22.8
30	32.6	25.5	22.7	19.2
July 2	30.9	25.7	22.4	21.0
4	26.8	29.2	22.2	21.8
6	28.7	23.9	25.7	21.7
8	26.7	27.0	25.0	21.3
10	26.6	23.4	26.2	23.8
12	25.4	23.5	27.2	23.9
14	33.0	24.6	19.2	23.2
16	31.8	26.7	21.2	20.3
18	28.3	26.3	24.2	21.3
20	24.4	29.3	23.7	22.5

data obtained from the crabs of Series A and the dots are the data from the crabs of Series C. The tidal maxima of Series C occurred six days prior to the tidal maxima of Series A at all times of day. The curves of Series C had no predominant peak after the crabs had spent one week in darkness because the melanin in the chromatophores of the *Uca speciosa* stayed nearly maximally dispersed throughout the day, rendering difficult an exact determination of the date on which the tidal maxima and minima of each portion of the day occurred. The identical situation was observed with *Uca pugnax* (Brown, Fingerman, Sandeen and Webb, 1953).

The tidal difference of six days in Figure 4 has been expressed as 4.9 hours. The latter value was determined from calculations based on the fact that the tides advance 48.8 minutes per day.

Inspection of the beach at Ocean Springs showed that the burrows of the *Uca pugilator* were found only in the sand of the open beach, whereas the burrows of the *Uca speciosa* were found only in the marsh grass from the high tide mark to the open sand. Figure 5 is a diagrammatic representation of the section of the beach at Ocean Springs where both species were collected.

TABLE VI

The average melanophore index for each of the 12 daily measurements of Series C, Uca speciosa

	8 A.M.	9	10	11	12	1 P.M.	2	3	4	5	6	7
June 16	2.8	3.7	3.7	4.0	4.1	3.7	3.6	3.8	3.6	3.4	3.4	3.0
18	3.7	3.7	3.8	3.6	4.1	4.4	4.4	4.6	4.4	4.3	4.1	3.3
20	3.5	4.3	4.3	4.5	4.5	4.5	4.7	4.7	4.7	4.6	4.6	3.9
22	4.4	4.8	4.8	4.8	4.6	4.6	4.5	4.4	4.5	4.6	4.5	4.1
24	4.3	4.7	4.7	4.6	4.8	4.7	4.6	4.6	4.6	4.5	4.2	4.2
26	4.4	4.6	4.6	4.6	4.4	4.5	4.4	4.5	4.4	4.5	4.5	4.3
28	4.4	4.7	4.8	4.8	4.8	4.8	4.8	4.6	4.8	4.6	4.6	4.4
30	4.6	4.7	4.7	4.6	4.6	4.7	4.6	4.6	4.5	4.6	4.4	4.5
July 2	4.0	4.3	4.7	4.7	4.7	4.8	4.7	4.8	4.8	4.7	4.7	4.6
4	4.6	4.8	4.6	4.7	4.7	4.7	4.6	4.7	4.7	4.7	4.4	4.4
6	4.8	4.7	4.8	4.8	4.7	4.8	4.7	4.8	4.7	4.7	4.7	4.6
8	4.7	4.7	4.9	4.8	4.8	4.7	4.8	4.6	4.6	4.7	4.5	4.4
10	4.5	4.6	4.7	4.7	4.7	4.6	4.7	4.5	4.6	4.5	4.4	4.3
12	4.7	4.8	4.8	4.8	4.7	4.6	4.6	4.7	4.7	4.5	4.7	4.3
14	4.7	4.9	4.8	4.9	4.8	4.7	4.6	4.5	4.6	4.5	4.5	4.3

Rarely did a *Uca speciosa* venture out of the marsh grass onto the open sand at low tide. On the other hand, at low tide the *Uca pugilator* left their burrows and moved to the water's edge to feed. The adaptive advantage of the tidal rhythm is thought to lie in the association between the time of low tide and the time at which the *Uca* leave their burrows to feed. The *Uca* at Ocean Springs are reported to leave their burrows to feed at low tide both day and night. Fiddler crabs living in a region of semi-diurnal tides leave their burrows to feed only when low tide occurs during daylight. If the fiddler crabs at Ocean Springs did not feed during a low tide at night, they would have to go without food for intervals of approximately 10 days when low tides occurred at night.

To explain the tidal difference between the two species of *Uca* the following

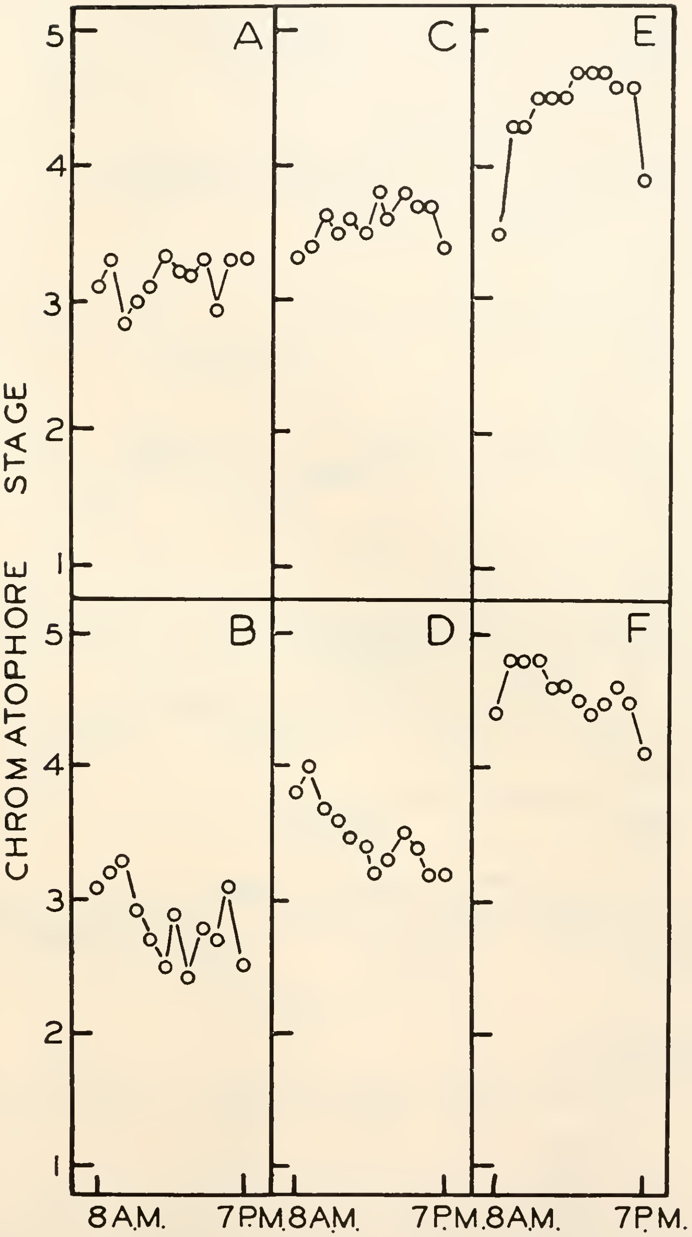

FIGURE 3. The daily patterns obtained on June 26 and 28 for Series A and B (*Uca pugilator*) and on June 20 and 22 for Series C (*Uca speciosa*). A and B in the figure are from Series A; C and D from Series B; E and F from Series C.

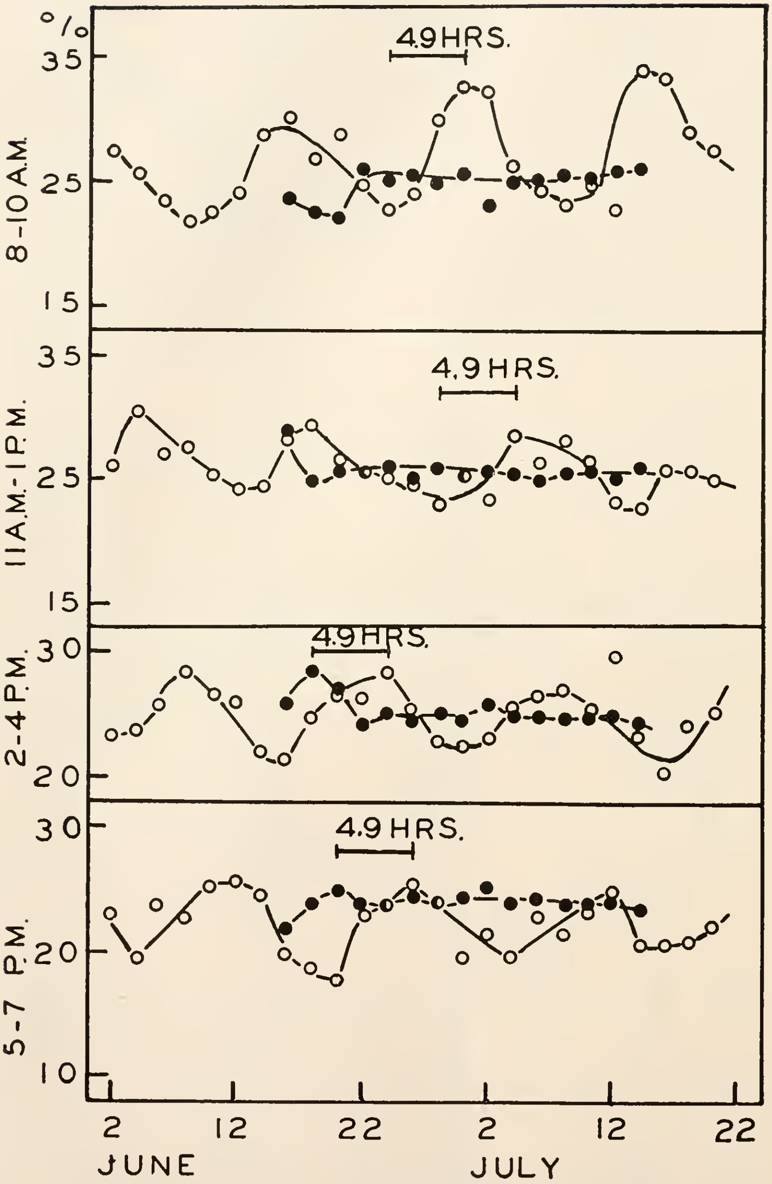

FIGURE 4. The relationship between the percentage of the total melanin dispersion occurring at each of four periods of the day and the day of the month. The circles represent the *Uca pugilator* collected on June 1 (Series A). The dots represent the *Uca speciosa* collected June 15 (Series C). The tidal curves of Series C are displaced six days to the left of the curves of Series A. In a tidal cycle, a displacement of six days is equivalent to 4.9 hours.

TABLE VII

The percentage of the daily melanin dispersion occurring at each of four periods of the day in Series C

	8–10 A.M.	11 A.M.–1 P.M.	2–4 P.M.	5–7 P.M.
June 16	23.4	28.6	26.0	22.1
18	22.5	25.0	28.6	24.0
20	22.3	25.7	27.2	24.8
22	25.8	25.8	24.4	23.9
24	25.2	26.1	25.4	23.3
26	25.4	25.2	24.7	24.7
28	24.7	25.9	25.4	24.0
30	25.5	25.3	24.8	24.4
July 2	23.0	25.7	26.0	25.3
4	25.2	25.5	25.2	24.1
6	25.2	25.2	25.0	24.6
8	25.6	25.6	24.9	24.0
10	25.2	25.7	25.2	23.8
12	25.7	25.3	25.1	24.0
14	26.0	26.0	24.4	23.5

hypothesis was developed. As the water begins to recede following a high tide, in effect a local low tide occurs for the *Uca speciosa* in their burrows among the marsh grass higher on the beach. This local low tide would occur earlier higher on the beach than would the low tide for the *Uca pugilator* in their burrows in the sand. The species living among the grass would, therefore, be free to feed earlier than the

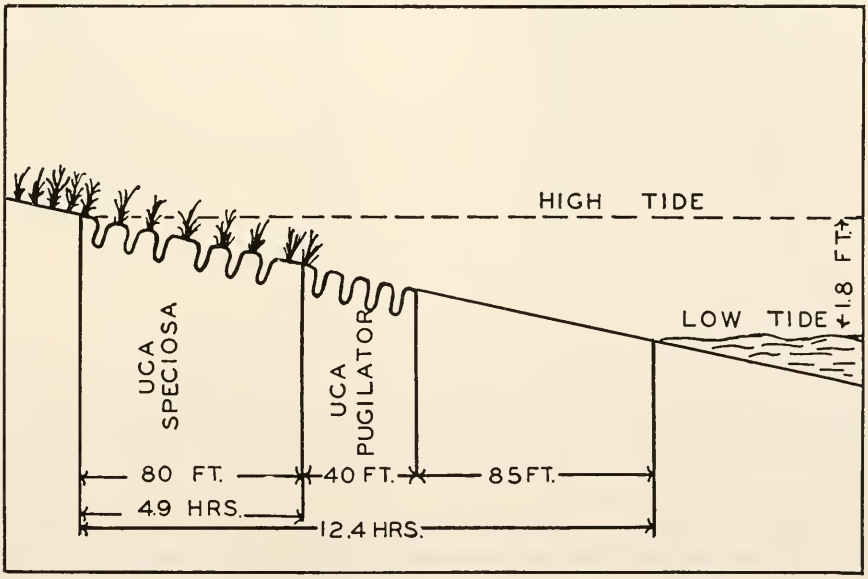

FIGURE 5. A diagrammatic representation of the section of the beach at Ocean Springs, Mississippi, where both species of *Uca* were collected.

species living in the sand. The earlier occurrence of a low tide in the marsh grass might, therefore, account for the *Uca speciosa* having a tidal rhythm 7.5 hours in advance of the tidal rhythm of the *Uca pugilator* whose burrows are in the sand.

To test this hypothesis measurements of the portion of the beach where both species were collected were made at low tide. To avoid bias, the measurements were made by an impartial local resident who was present when the collections were made on June 15. This resident was given a sketch of the beach. On this diagram were indicated the regions of the beach to be measured. He did not know how the data he obtained were to be treated nor did he know a 4.9 hour tidal difference between the species had been found in the laboratory. The measurements have been incorporated into Figure 5. The width of the beach from the high tide mark to the low tide mark is 205 feet. The distance from the high tide mark to the first *Uca pugilator* burrows in the sand is 80 feet. Twelve and four-tenths hours are required for the water to move from the high tide mark to the low tide mark. The time required for the water to recede the 80 feet from the first *Uca speciosa* burrows to the first *Uca pugilator* burrows was then calculated. This value is 4.9 hours, close to the result observed in the laboratory for the tidal difference in hours between the two species.

TABLE VIII

The amplitude of the daily rhythm of animals maintained in darkness. The amplitude is expressed as the total of the average stages for the 12 daily measurements of the melanophores

	Series A	Series B	Series C
June 2	34.2		
4	34.5		
6	38.2		
8	40.2		
10	43.6		
12	42.9		
14	44.4		
16	39.2	35.9	42.8
18	40.0	39.5	48.4
20	39.0	41.5	52.8
22	39.5	41.8	54.6
24	38.3	42.6	54.5
26	37.8	42.9	53.7
28	34.1	41.8	56.1
30	37.0	37.5	55.1
July 2	29.7	39.2	55.5
4	33.4	40.4	55.6
6	33.5	39.2	56.8
8	33.2	41.6	56.2
10	33.9	41.0	54.8
12	31.9	38.8	55.9
14	28.2	34.4	55.8
16	25.2	33.7	
18	26.8	36.0	
20	29.3	38.2	

A semilunar rhythm of amplitude of the daily pigment dispersion

The sums of the twelve daily average chromatophore indices for Series A, B, and C have been compiled in Table VIII and depicted in Figure 6. The amplitudes for the three series increased from the outset of the observations. The amplitude of the daily pigmentary rhythm of the *Uca pugilator* of both Series A and B then decreased noticeably. Increases and decreases followed one another. Closer inspection showed this increase and decrease of amplitude was semilunar in nature. The minima occurred 15 days apart which is the approximate frequency of a semilunar rhythm. Furthermore, these minima were observed on the same days that

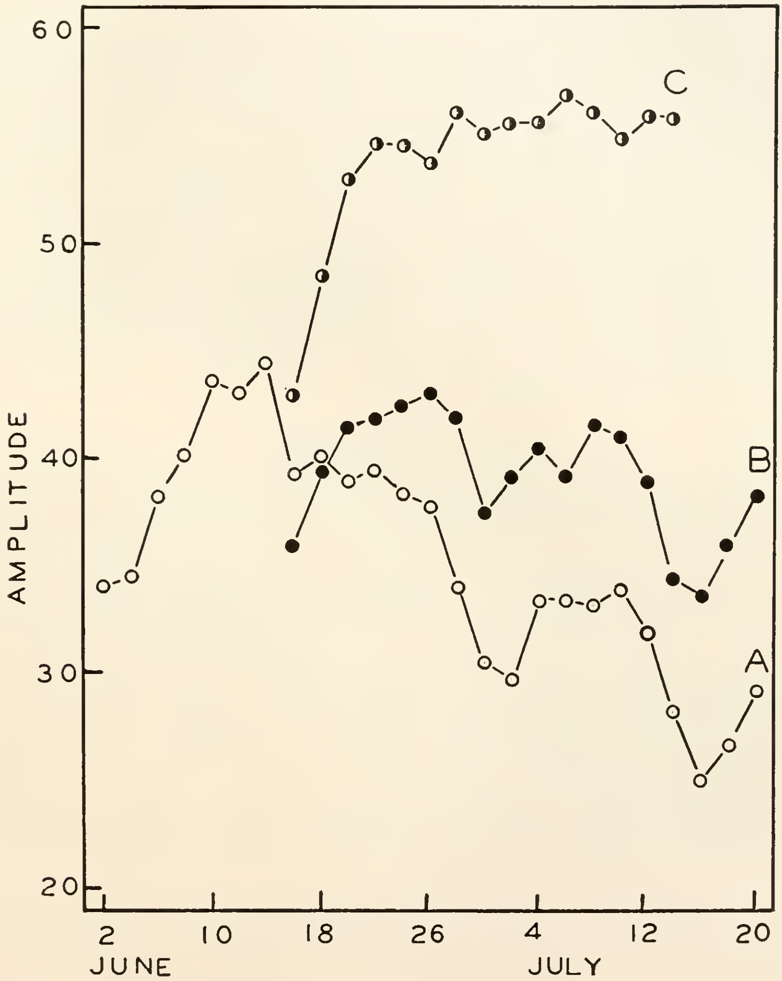

FIGURE 6. The amplitude of the daily patterns for Series A, B, and C. The amplitude has been expressed as the sum of the 12 average chromatophore stages determined every second day for each series. Series A and B are *Uca pugilator*. Series C is *Uca speciosa*.

the tidal maxima occurred in the 8–10 A.M. portion of the day (Figs. 2, 4, and 6). Closer inspection of the data from Series C also disclosed a semilunar rhythm of amplitude which was not as pronounced as the rhythm of Series A and B because of the tendency of the melanin in the chromatophores of the *Uca speciosa* to remain nearly maximally dispersed after a week in darkness. The minima of Series C occurred 5.5 days ahead of the minima of Series A and B, again demonstrating the phase difference between the species.

The days on which the minima of amplitude occurred coincided with the days on which the 8–10 A.M. portion of the daily curve was maximal (compare Figures 2 and 4 with Figure 6). To produce a maximum at 8–10 A.M. the P.M. tide must progress late into the evening and therefore has no ability to cause a supplementary dispersion of the pigment in the afternoon. The A.M. tide, 12.4 hours after the P.M. tide, is still too early to have an effect upon the amplitude. The daily rhythmicity alone keeps the pigment semi-dispersed in the late afternoon on the days with a late evening tide. As the A.M. tide progresses through the early morning hours toward the center of the day the amplitude of the entire daily curve is raised by the tidal influence and the 8–10 A.M. portion of the curve is maximal. The amplitude begins to decrease again as the tide progresses into the late afternoon and early evening. Then the daily rhythm alone causes the dispersion of the melanin during the morning hours.

General Discussion

The tidal rhythm determined for both *Uca pugilator* and *Uca speciosa* is similar in form to the tidal rhythm previously determined for *Callinectes sapidus* and *Uca pugnax* despite the fact the specimens of the latter species were taken from a region exhibiting only semi-diurnal tides. Originally this endogenous tidal rhythm was probably induced by a rhythmic environmental factor with a 12.4 hour frequency. This tidal rhythm has now become such a deep-rooted phenomenon that it persisted even after the crabs had been placed in an environment that was constant with respect to the rhythmic environmental factors. The necessity of the two events spaced 12.4 hours apart having been similar to one another was apparently secondary to the frequency which became impressed upon the crabs. If the frequency were of secondary importance to the nature of the events, then *Uca* living in a habitat with diurnal tides would have exhibited a rhythm with a frequency which is twice that observed; the tidal maxima would have been found to be 29.5 days apart.

The tidal phase difference between the two species living at different levels on the same section of beach was entirely unexpected. The occurrence of this difference demonstrates that the tidal rhythm has more adaptive significance than was suspected previously. The phases of the tidal rhythm are apparently set by the tides within an extremely limited strip of beach. According to the authorities at the Gulf Coast Research Laboratory only within the past year have the *Uca* invaded the portion of the beach where they were collected. The phases of their tidal rhythms were possibly reset in accordance with the influence of the local tides near their burrows within the past year.

The morphological difference in the melanophore pigment pattern of the two species may be a partial explanation for the semilunar rhythm of daily amplitude being more apparent in *Uca pugilator* than in *Uca speciosa.* When the pigment in the melanophores of *Uca pugilator* was completely dispersed (stage 5) the melanophores were still discrete entities. Light portions of the exoskeleton could be seen between the melanophores. However, when stage 5 was attained by the melanophores of *Uca speciosa* the melanophores lost their individuality and the entire leg was homogeneously black. How this difference in morphology is translated into the difference in behavior is not readily apparent.

The chromatophore behavior of *Uca speciosa* is similar to the pigmentary behavior of *Uca pugnax.* The melanophores of both species darkened the entire leg when the pigment was completely dispersed. The chromatophores of both species also tended to remain completely dispersed throughout the day after a similar length of time in darkness.

The tidal rhythm of the *Uca pugilator* of Series A was determined in the laboratory over a period of 48 days which is the longest interval any group of *Uca* has ever been observed. Throughout this period there was no indication that the frequency of the tidal rhythm was changing.

Summary and Conclusions

1. The pigment in the melanophores of the fiddler crabs, *Uca pugilator* and *Uca speciosa,* showed a persistent tidal rhythm with a frequency of 12.4 hours.
2. The nature of the tidal rhythm of both species was similar to the tidal rhythms previously reported for the fiddler crab, *Uca pugnax,* and the blue crab, *Callinectes sapidus.*
3. No detectable difference in the character of the pigment migration was apparent at times of low and high tide which occur 12.4 hours apart where these two species of *Uca* were collected.
4. The tidal rhythm of *Uca speciosa* was six days behind the tidal rhythm of *Uca pugilator.* This difference is equivalent to 4.9 hours in a tidal cycle.
5. An hypothesis is presented to explain this tidal difference. The difference in the habitat of the two species of *Uca* on the same beach, the width of the beach, and the time required for the water to recede from the burrows of the *Uca speciosa* to the burrows of the *Uca pugilator,* which live at a lower level of the beach, are the factors which have been considered in formulating the hypothesis.
6. A semilunar rhythm of the amplitude of the daily dispersion of the pigment in the melanophores of both species was found.
7. The possible adaptive significance of the phase difference of the tidal rhythms of the two species is discussed.

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THE LOCI OF CONTACT CHEMORECEPTORS INVOLVED IN FEEDING REACTIONS IN CERTAIN LEPIDOPTERA [1]

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In our review (1949) of literature on the loci of contact chemoreceptors of insects we listed 10 species of butterflies which had been shown experimentally to have contact chemoreceptors on the tarsi. It was assumed that these insects also had receptors on the mouth-parts or in the mouth, but this had not been shown experimentally. Up to that time, four species of moths had been tested for possible possession of tarsal chemoreceptors and found not to possess them. At that time, we tested three species of butterflies, two of which had been shown previously to have tarsal chemoreceptors, located these more exactly and found that the terminal part of the proboscis in each is sensitive to chemical stimulation by solutions. The antennae and palpi were found not to be loci of contact chemoreceptors sensitive to sucrose solutions. The contact chemoreceptors on the tarsi and proboscis are involved in feeding reactions, and these should probably be so designated to differentiate them from chemoreceptors possibly involved in mating (Roth and Willis, 1952) or oviposition (Dethier, 1947).

As an extension of the earlier work, we have located the trophic contact chemoreceptors of four species of butterflies, only one of which has been tested previously, and two species of moths, neither of which has been tested previously.

MATERIALS AND METHODS

The following species of butterflies were studied. The scientific names are those given by Klots (1951).

Family: Satyridae
    Cercyonis pegala—The Wood Nymph or Grayling
        subspp. *alope* and *nephele*
        20 individuals, captured in the field
    Lethe eurydice—The Eyed Brown
        3 individuals, captured in the field
Family: Nymphalidae
    Speyeria cybele—The Great Spangled Fritillary
        2 individuals, captured in the field

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Limenitis arthemis—The White Admiral
2 individuals, captured in the field

The following species of moths were tested. The scientific names are those given by Borror and Delong (1954).

Family: Amatidae (Syntomidae, Ctenuchidae, Euchromiidae)
Ctenucha virginica
22 individuals, captured in the field
Scepsis fulvicollis—The Yellow-collared Scape Moth
15 individuals, captured in the field

In the first and last two species, these were approximately half males and half females. *Cercyonis pegala* was found by Anderson (1932) to have tarsal chemoreceptors; the others have not previously been tested. Reporting results obtained with the small number of individuals of the last three butterflies in the list is justified only because the responses were so clear-cut and so similar to those of the other related species. The moths are day-flying and feed on nectar, and thus differ in habits from the usual moths. They have well developed proboscides.

The butterflies were immobilized for testing as described by Minnich (1921, 1922a, 1922b) by clamping the wings together with spring clothes-pins. The moths were mounted by fixing the dorsum of the prothorax and the wings to wax blocks on the ends of glass rods, as described by Frings (1947). Thus fixed, the animals could easily be handled and observed.

The contact chemoreceptors were located as described previously (Frings and Frings, 1949). Series of tests of variable duration and number were made each day on the fixed animals. Before each series the insects were given all the water they would drink. These butterflies and moths proved to be avid drinkers, and often kept the proboscis extended for more than 10 minutes. A 1 M sucrose solution in water was used for most of the tests, and contact of this with the receptors elicited uncoiling of the proboscis and feeding if allowed. The proboscis response could thus be used as the index of reception. Temperatures were uncontrolled and varied from 18°–25° C.

For general location of the receptors, the part to be tested was either immersed in the sucrose solution in a small dish or the solution was brought to the part on small artists' brushes. Each test with the solution was preceded by one or more control tests with water. If at any time the insects responded to water, they were allowed to drink until sated, were thereupon put aside for a time, and were retested later.

For more specific location of the receptors, two methods were used. Local stimulation of suspected parts with droplets of sucrose solution on finely drawn, glass microneedles was used with uninjured insects. These tests were also preceded by control tests with water. Operations on the animals for removal of suspected or known loci and later testing were also performed, with paired controls similarly treated except for the removal of parts. Generally, the insects were unanaesthetized for these manipulations. The tests were carried out under a binocular dissecting microscope at up to 30 × magnification.

For microscopic examination of possible end-organs, structures were removed to 70% ethyl alcohol, transferred to 95% alcohol and mounted on slides in Diaphane.

RESULTS

In these butterflies, the rudimentary fore-legs characteristic of the Nymphaloids, the palpi, and the antennae seem to lack receptors for sucrose. The antennae are quite sensitive to water vapor if the animals are thirsty. Receptors are present on the ventral sides only of the tarsi of the mesothoracic and metathoracic legs and on the distal portion of the proboscis, extending about ¼ of the length of the proboscis from the tip. With *C. pegala* and *L. eurydice,* but not with the other two species, local stimulation techniques showed that the three distal segments of the mesothoracic and metathoracic tarsi have receptors and the basal segment does not. The second segment of the mesothoracic tarsus in *C. pegala* definitely has the receptors along its entire length, while that of *L. eurydice* has them only at the distal end. Tests on the second segment of the metathoracic tarsus of *C. pegala* gave inconclusive results, while tests on this segment of *L. eurydice* gave definitely negative results. A total of 51 paired tests (H_2O vs. sucrose solution) were made in 10 days.

Ctenucha virginica proved to be an easy animal to study. A total of about 250 paired tests were made in 10 days. All the experimental methods were used. The palpi lack contact chemoreceptors mediating proboscis extension and the antennae seemed to lack them in the early tests. All the tarsi possess the organs. On the prothoracic and mesothoracic tarsi they are on the ventral sides of all five of the tarsal segments, but not on the distal parts of the tibiae. On the metathoracic tarsi, the receptors are on the ventral sides of the three terminal segments, probably on the second segment, and probably not on the basal segment and the tibia. The distal ¼ to ⅕, only, of the proboscis proved to be sensitive when tested with glass microneedles bearing 1 *M* sucrose solution after control tests with water. When the tarsi or the tip of the proboscis was touched with a 1 N NaCl solution, the animal drew the proboscis into a tighter coil. If the animal was feeding on sucrose solution, touching the tip of the proboscis with NaCl solution caused immediate recoiling of the proboscis. Thus these receptors allow differentiation between acceptable and unacceptable solutions.

Scepsis fulvicollis also proved to be easy to test, and all the methods for locating the receptors were used. A total of about 400 pairs of tests were made in 16 days. Two additional methods were also used with these. First, the insects were tested with saturated sucrose solution as well as 1 *M* solution. Second, after tests on the legs had been made, all the legs were removed and the antennae and palpi tested. In all the previous tests, the antennae and palpi were tested with the animals intact or with only the prothoracic or prothoracic and mesothoracic legs removed.

The palpi seem not to bear receptors mediating the proboscis response. The ventral surfaces of the three pairs of tarsi bear end-organs. On the fore-leg, the receptors are on all five segments of the tarsus, but not on the tibia. On the mid-leg, the distal four segments of the tarsus definitely bear receptors. When tested with 1 *M* sucrose solution, the basal segment seemed not to bear the receptors, but when tested with saturated sucrose solution definite positive responses were obtained. The tibia seemed not to have receptors. The hind-leg showed a similar situation. When tested with 1 *M* sucrose solution, only the three distal segments of the tarsus seemed to have receptors, but when tested with saturated sucrose solution all five of the segments proved to bear them. The tibia again seemed not to have these receptors. The distal ⅕ to ⅙ of the proboscis also proved sensitive to sucrose solutions; uncoiling was elicited on contact with a needle moistened with the sucrose solutions. The other parts of the proboscis could be touched with sucrose solutions without eliciting uncoiling. NaCl solution, as in *Ctenucha,* elicited a rejection reaction.

The situation with respect to the antennae in *Scepsis* was interesting. As long as the animals had tarsi, the antennae seemed to lack the receptors. Only occasionally would the insect uncoil the proboscis when sugar-water touched the antennae, and usually the insect would then take water and afterward be negative to sugar-water. If only the hind tarsi or even one hind tarsus alone were present, the reaction to antennal contact indicated lack of receptors. When all the legs were removed, however, the antennae seemed receptive, and the differential response to sucrose solution became almost perfect. By the time that this discovery was made, the individuals of the other species had died, and it was impossible to test them similarly.

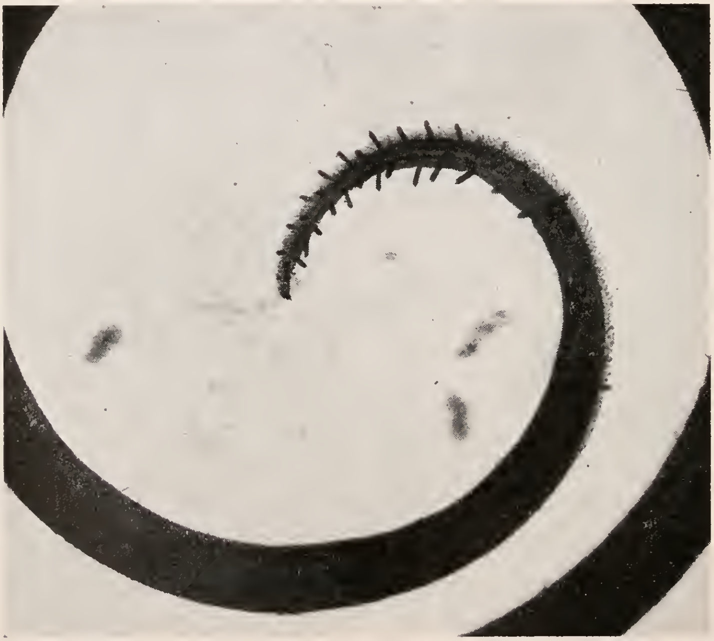

FIGURE 1. Terminal portion of proboscis of *Ctenucha virginica* (× 70).

The previous experiments were made during the summer of 1955. In January, 1956, adults of *Ctenucha virginica* were obtained from caterpillars reared from eggs laid during the past summer. Six of the adults (3 ♂♂ and 3 ♀♀) were tested and found to have antennal contact chemoreceptors. When either or both of the first two pairs of legs were present, no consistent reactions were obtained on stimulation of the antennae with sucrose solutions. When the first two pairs of legs were removed, leaving only the metathoracic legs, stimulation of the antennae with sucrose solutions consistently evoked the feeding response. Smyth (personal

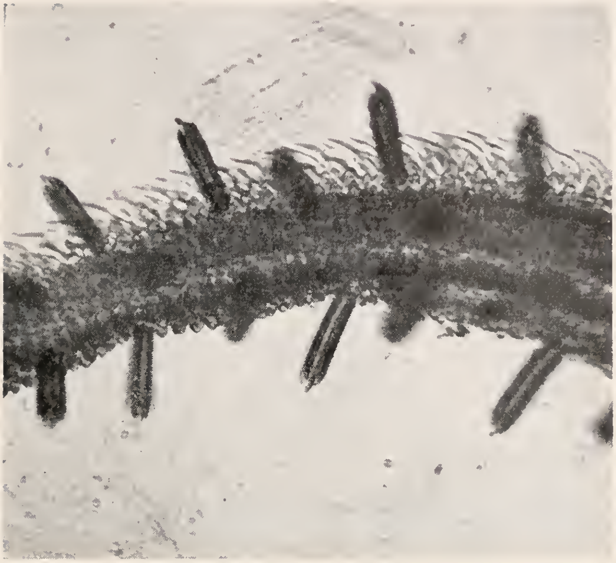

FIGURE 2. Section of terminal portion of proboscis of *Ctenucha virginica* (× 350) to show probable chemoreceptive sensilla. The convex side has a rather dense layer of non-sensory epicuticular "hairs."

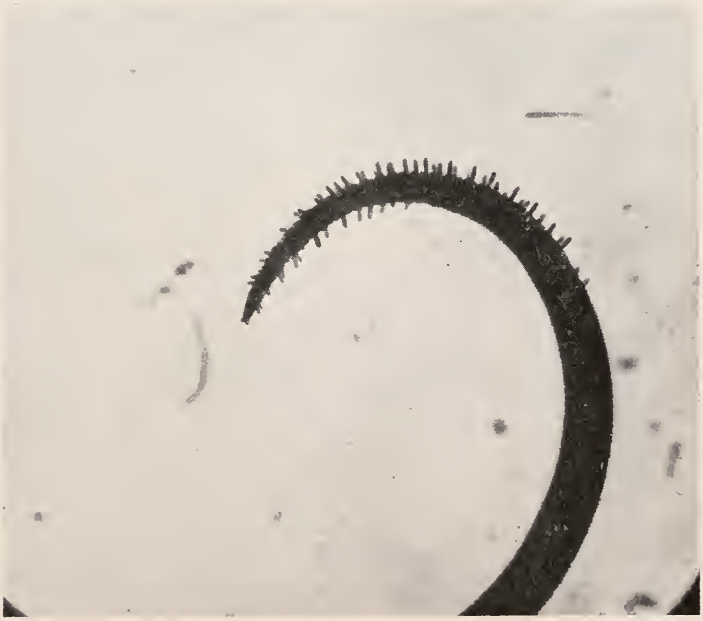

FIGURE 3. Terminal portion of proboscis of *Scepsis fulvicollis* (× 70).

communication) has recently recorded discharges from receptors on the antenna of *Scepsis* when they were stimulated with sucrose solutions, thus confirming electrophysiologically the results of the behavioral experiments.

Morphological studies on the tarsi of these Lepidoptera have not enabled us to select any particular sensilla as the end-organs. There are present, as in *Danaus plexippus* (Frings and Frings, 1949), short, thin-walled trichoid sensilla on the ventral surfaces of the tarsi among other stouter hairs and spines. These could be the receptors, but there is no proof of this.

On the proboscis of the moths studied, at the terminal portion found by experiments to be sensitive, there are many large sensilla (Figs. 1–4) that are almost certainly the contact chemoreceptors. These have a peculiar structure, appearing something like candles. There is a shaft, which is polygonal in cross-section, 6–7-sided in *Ctenucha* and 5–7-sided in *Scepsis,* with the angles seemingly thickened. This is capped with a thin-walled nipple-shaped structure, around the base of which the thickened corners of the shaft extend as points. In *Ctenucha* these range in size from 45–50 μ long and 12 μ thick at the proximal end of the group to 35–40 μ long and 12 μ thick near the tip, most being 40–45 μ long and 12 μ thick. At the tip there is a small group of shorter organs 20–25 μ long and 12 μ thick. In *Scepsis* the organs are somewhat smaller: 40–45 μ long and 9 μ thick at the proximal end decreasing regularly in size toward the tip to 25–30 μ long and 7 μ thick, most being 30–40 μ long and 7 μ thick. The organs in the group at the tip measure 15–18 μ long and 7 μ thick. The exact designation that one should give to these sensilla is not clear. Are they modified trichoids or basiconics? Since no other obvious sensilla appear at this region of the proboscis and these correspond in distribution

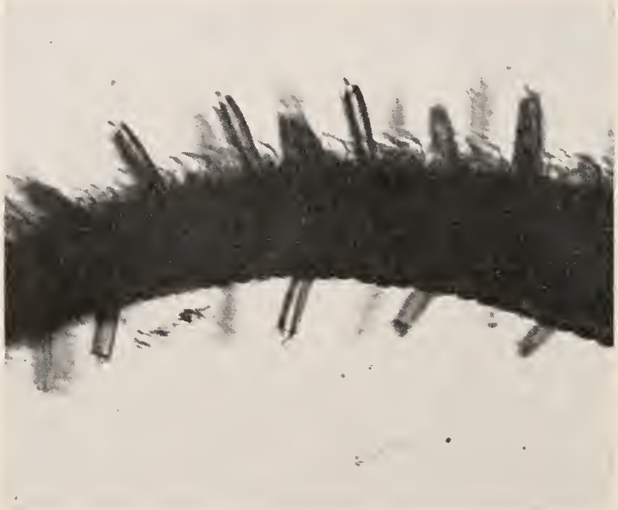

FIGURE 4. Section of terminal portion of proboscis of *Scepsis fulvicollis* (× 350) to show probable chemoreceptive sensilla. The epicuticular "hairs" on the convex surface are shorter than in *Ctenucha.*

with the experimentally determined sensitive region, they are probably the end-organs.

Among the butterflies, we have examined only the proboscis of *C. pegala*. The distal $\frac{1}{5}$ of it bears similar organs, 30–35 μ long and 7 μ thick, varying little in size throughout. These correspond in position also with the experimentally determined contact chemoreceptors and are thus probably the end-organs.

DISCUSSION

The results of this study and those of previous works (Minnich, 1921, 1922a, 1922b; Anderson, 1932; Weis, 1930; Frings and Frings, 1949) indicate that the butterflies (Superfamily: Papilionoidea) generally have contact chemoreceptors sensitive to sucrose and mediating feeding responses (trophic contact chemoreceptors) on the tarsi and distal part of the proboscis. The following species have been shown experimentally to possess them:

- Family: Satyridae
    - *Lethe eurydice*
    - *Cercyonis pegala*
- Family: Danaidae
    - *Danaus plexippus*
- Family: Nymphalidae
    - *Speyeria cybele*
    - *Limenitis arthemis*
- Family: Pieridae
    - *Colias philodice*
    - *Pieris rapae*

The following species have been shown to have contact chemoreceptors on the tarsi and almost certainly on the proboscis, but the latter has not yet been shown experimentally.

- Family: Lycaenidae
    - *Lycaena thoe*
- Family: Nymphalidae
    - *Phyciodes tharos*
    - *Nymphalis antiopa*
    - *Vanessa atalanta*
    - *Limenitis archippus*
- Family: Papilionidae
    - *Papilio machaon*
    - *Papilio polyxenes*

In the Superfamily Hesperioidea (Skippers), *Polites mystic* has been reported by Anderson (1932) as showing only questionable discrimination by the tarsi between sucrose solutions and water. The proboscis was not tested. From preliminary observations on eight individuals (*Hesperia* or *Polites* spp.) it seems highly probable to us that tarsal and proboscidal receptors are present, but more critical tests are needed. These Lepidoptera, as noted by Anderson, are erratic in response, often going for three or four days without taking water or sucrose solutions.

Among moths, Weis (1930) has reported that the following species lack tarsal chemoreceptors; other parts were untested.

Family: Sphingidae
    Macroglossa stellatarum
    Acherontia atropos
    Sphinx pinastri
Family: Saturniidae
    Aglia tau

Our results show that the following have tarsal, proboscidal and antennal receptors.

Family: Amatidae
    Ctenucha virginica
    Scepsis fulvicollis

The situation in moths needs much more study with many more species from a number of families. Many species of moths are known to have rudimentary mouthparts and to pass the adult life without feeding; it seems hardly likely that they would have contact chemoreceptors. The habits of the sphingid moths would seem to limit the utility of tarsal chemoreceptors. The day-flying, flower-feeding habits of the Amatidae make their sensory requirements like those of butterflies. A broader study might indicate how far this seeming adaptation of chemoreceptors to habits is found.

The results with the antennae of *Scepsis* and *Ctenucha* clearly mean that further testing is necessary before it is safe to conclude that antennal receptors are not present in other Lepidoptera. It is obvious that the conditions under which tests of contact chemoreception are made must be carefully specified. It seemed earlier (Frings and Frings, 1949) that antennal contact chemoreceptors were present only in Hymenoptera. Since then, Roth and Willis (1952) have reported them in cockroaches. They used sexual reactions rather than feeding reactions. The change in behavioral index of reception thus changed the interpretation of the location of the receptors. With the legs present, *Scepsis* and *Ctenucha* seemed to lack antennal trophic contact chemoreceptors. With the legs removed, they seemed to be present. With 1 *M* sucrose solution, *Scepsis* seemed to have receptors only on the distal segments of the mesothoracic and metathoracic tarsi. But with saturated sucrose solution, all segments were clearly sensitive. Regardless of theoretical interpretations one wishes to make of these cases, the necessity for exact specification of conditions of testing is obvious. All the receptors available to the animals may not be used in any specific behavioral situation (Tinbergen, 1951, pp. 25–37), or the receptors used in a particular behavior pattern may have different thresholds. Further, we may conclude that, while positive reactions in tests such as these indicate the presence of contact chemoreceptors, negative reactions do not necessarily show their absence. Negative results must be interpreted with great caution.

SUMMARY

1. The loci of contact chemoreceptors, stimulation of which by sucrose solutions elicits proboscis extension, were determined for the butterflies: *Cercyonis pegala* and *Lethe eurydice* of the Satyridae, and *Speyeria cybele* and *Limenitis arthemis* of the Nymphalidae; and the diurnal moths: *Ctenucha virginica* and *Scepsis fulvicollis* of the Amatidae.

2. Local contact and excision methods were used. All have the receptors on the terminal part of the proboscis and on all the functional tarsi. The palpi probably do not bear the receptors, and in the butterflies the antennae likewise. In *Ctenucha* removal of the first two pairs of legs and in *Scepsis* removal of all the legs resulted in feeding reactions on stimulation of the antennae with sucrose solutions.

3. These results emphasize the need for careful control and specification of testing conditions and for cautious interpretation of negative results in experiments such as these.

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ON REGULATION OF CONCENTRATION AND CONTENT OF CHLORIDE IN GOLDFISH

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Only little is known about the variations which may normally occur in the content of water and ions of fresh water fishes. Such variations are determined by the rates at which water and ions are taken up or lost. Water is continuously being absorbed through the outer surfaces, various ions may be taken up actively by the gills or ingested with the food. Loss takes place through the kidneys and the surface. The renal loss varies with the urine flow and ionic concentration of the urine which is generally very dilute. Loss of ions by diffusion through the skin also varies. It is for instance strongly increased in disturbed or wounded fish (Krogh, 1937; Meyer, 1948).

The capacity for uptake of sodium and chloride ions from dilute solutions is highly developed in many fresh water fishes (Krogh, 1937, 1939; Meyer, 1948, 1951; Mullins, 1950; and Wikgren, 1953). In the stickleback *Gasterosteus aculeatus* Mullins showed that one quarter of the amount of sodium in the body is exchanged per day. The osmotic and ionic concentration is, however, kept rather constant despite a high rate of turn-over of ions. The osmotic pressure is about 300 m.osM./l in fresh water teleosts (Duval, 1925; Krogh, 1939; Vinogradov, 1953; Robertson, 1954). Variations in content of sodium and chloride ions—if they occur to any degree—are therefore presumably accompanied by such variations in water content that the osmotic pressure of the body fluid remains constant.

In the present investigation we followed during several weeks the daily changes in chloride content and weight of undisturbed starving goldfish. From these data it was possible to estimate daily and long-term changes in osmotic pressure and in total content of water and chloride of the body. The chloride content of the body was also measured by the dilution method using the radioactive isotope Cl^{36}. Changes in chloride content could thus be compared with the total amount of exchangeable chloride in the fish.

TECHNIQUE

Healthy, 10–20 g., goldfish were used. They were kept in aerated chambers of the type previously described (Jørgensen, Levi and Ussing, 1946). The experiments were performed during the months of March to May in an unheated room where the temperature varied from 15 to 20° C.

Daily variations in chloride content of the fish were calculated from changes in chloride content of the medium, which was 200 ml. tap water, of initial concentration about 1–2 m.eq./1. Chloride was determined in one-ml. samples according to Rehberg in Schnohr's (1934) modification. The chloride content of the medium was estimated with an accuracy of about 5 μeq. Only changes amounting to some 10 μeq. and more in chloride content are therefore significant.

Great care was taken not to disturb the fish unduly during the weighings, because handling itself can induce loss of weight and of chloride. The fish, which apparently got accustomed to the weighing procedure, were carefully transferred from their chambers into nets which were drained on filter paper. From the nets the fish were put into weighing-glasses without being touched directly by the hands. Weighings were made in duplicate. The fish were returned to water between the two weighings which were thus performed under the same conditions. The weight in grams was read with two decimals and rounded to 0 or 5. The duplicates deviated maximally by 100 mg. Daily changes in weight should therefore amount to 0.2 g. or more in order to be significant.

Total exchangeable chloride was determined in three well-nourished goldfish which were kept in 200 ml. tap water enriched with Cl^{36}-labelled sodium chloride. Changes in specific activity of the chloride in the medium were followed. From the degree of dilution of Cl^{36} the amount of chloride in medium plus goldfish was computed. Radioactivity was measured on evaporated 0.5-ml. samples of the medium by means of a Geiger-Müller end-window counter.

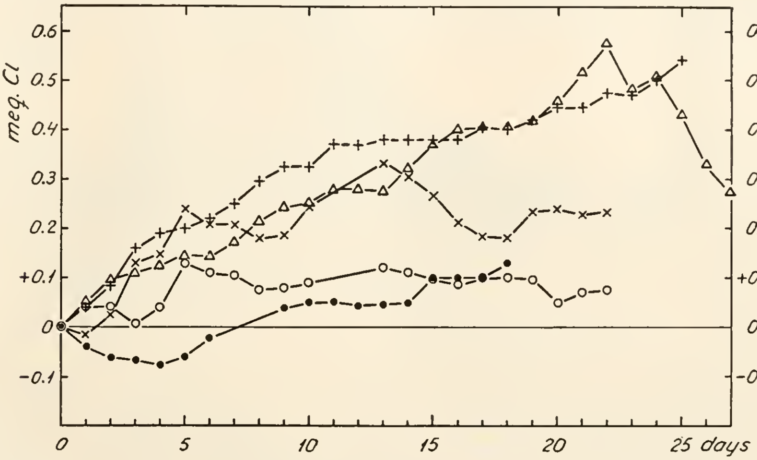

FIGURE 1. Spontaneous changes in chloride content of 5 starving goldfish, initially weighing 11–19 g.

RESULTS

Variations in chloride content were followed during several weeks in five goldfish. As may be seen from Figure 1, uptake or loss of chloride often continued for several days or even weeks at a time, resulting in large changes in total chloride content of the body. One fish, weighing 19 g., took up 600 μeq. of chloride in 27 days, and another one, weighing 17 g., took up 575 μeq. in 22 days. Total exchangeable chloride, determined in three fed goldfish, was found to be 34.5, 35.5 and 43.0 μeq./g. body weight. Assuming an initial chloride content of 38 μeq./g., the two above mentioned fish thus increased their chloride content by 83 and 89 per cent, respectively.

Even the day-to-day changes in chloride content can be considerable. All avail-

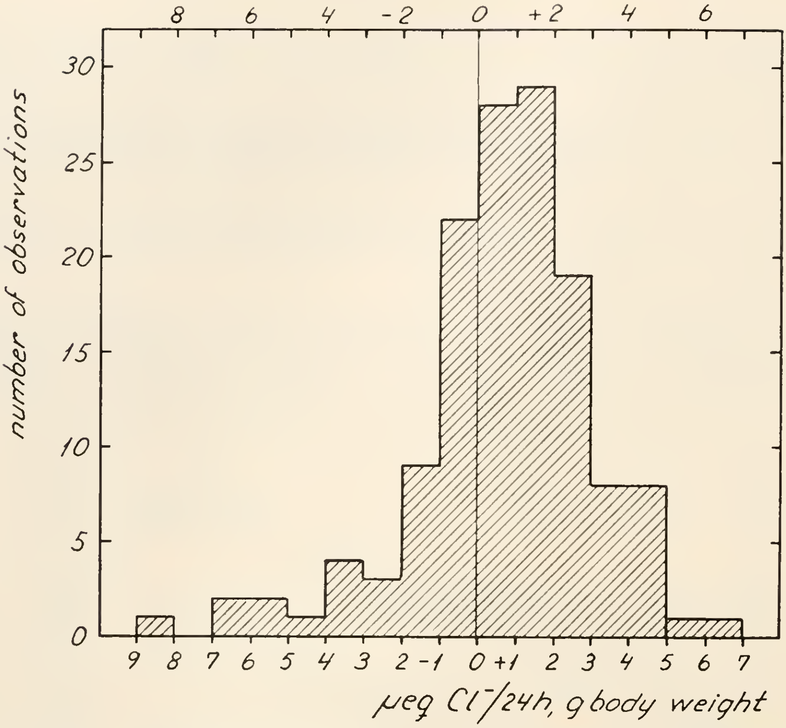

FIGURE 2. Spontaneous daily changes in chloride content of 9 starving goldfish.

able observations, 138 in number, on spontaneous daily changes in chloride content of nine goldfish are given in Figure 2, expressed in μeq. per gram of body weight. Maximum daily gains of 6–7 μeq. per gram correspond to $6.5/38 = 17$ per cent change in chloride content and maximum loss of 8–9 μeq. to some 22 per cent. Without proportional change in water content such large variations in chloride content would result in a strongly varying osmotic pressure. The daily changes in weight were therefore measured simultaneously with the daily changes in chloride content in order to estimate how constantly the osmotic pressure was maintained. The animals were adapted to the experimental conditions for ten days prior to the

TABLE I

Regression of daily weight changes (g.) of starving goldfish on daily changes in chloride content (m.eq.)

Experiment No.	Regression equations	Correlation coefficients	Statistical significances	Maximal daily changes in Cl content (μeq.)
1	$y=6.3x-104$	0.719	$0.02>P>0.01$	$-45+23$
2	$y=6.7x-112$	0.762	$0.01>P>0.001$	$-53+54$
3	$y=4.2x-97$	0.645	$0.05>P>0.02$	$-46+41$
4	$y=1.4x-108$	0.320	$0.3>P>0.2$	$-9+46$
5	$y=5.1x-216$	0.867	$P<0.001$	$-87+64$

measurements which were extended over a period of 11–15 days. A statistically significant correlation was found between daily chloride and weight changes in four of the five goldfish under experiment (Table I). In experiment 4 without significant correlation the daily variations were small. It may be seen from the table that the larger the daily changes the more significant was the correlation.

The regression coefficients in experiments 1, 2, 3 and 5 are 4.2, 5.1, 6.3 and 6.7. The mean value is 5.6. Thus, a change in chloride content of one m.eq. was accompanied by a change in weight of 4.2 to 6.7 g. Or in other words retention or excretion of 150 to 238 μeq. Cl was followed by retention or excretion of one ml. water.

The regression equations further show that in the five goldfish a chloride-independent loss of weight took place, ranging from 97 to 216 mg. daily. Computed per 100 g. body weight the values are 0.64, 0.75, 0.87, 0.88 and 1.34 g. which probably mainly correspond to the daily averages of break-down of tissue due to the metabolism.

Discussion

In the goldfish and other fresh water fish, chloride can be taken up through the gills and ingested with the food or by drinking. It might be of interest to estimate the relative importance of these sources. From Figure 2 it can be seen that in some 25 per cent of the periods of observation from 200 to 700 μeq. Cl was gained per day and 100 g. body weight. The metabolic rate was measured in three of the fish under experiment. Oxygen was determined by means of the Winkler technique. Measurements of the oxygen consumption were performed on undisturbed fish which were not removed from their experimental chambers; 0.233, 0.240 and 0.237 l. of oxygen was consumed per 24 h. and 100 g., corresponding to a combustion of about ¼ g. mixed organic matter. Even when the metabolic rate of starving fish is lower than normal probably less than one gram of organic matter is needed daily in order to cover the energy requirements. If one gram were to be obtained in the form of animal food containing, say, 70 per cent water and 40 μeq. Cl/g., this would correspond to an ingested amount of 130 μeq. Cl per 24 h. and 100 g., or only a quarter of an amount which is easily taken up through the gills. Presumably much less than 40 μeq. Cl/g. is contained in the food of the goldfish which eat much plant material, partly decaying, and poor in chloride.

Recent investigations of Allee and Frank (1948, 1950) show that some drinking takes place in fresh water fishes. Chloride uptake by drinking can, however, only be negligible in the goldfish. The daily diuresis amounts to some 10 ml. per 100 g. Most of this water is probably taken up through the outer surface, especially the gills (Krogh, 1939). Thus, at most a few μeq. Cl can be obtained by drinking in ordinary fresh water which generally contains less than one m.eq. Cl per liter. Under normal conditions, therefore, the gills must be more important organs for absorption of chloride than is the alimentary tract.

The total amount of chloride in the body is not precisely regulated in the goldfish, at least not in starving animals, whose chloride content could be almost doubled. The chloride concentration and osmotic pressure are better regulated. A loss or gain of only some 0.3 m.eq. Cl/100 g. within 24 hours generally coincides with a corresponding loss or gain of water (Table I). An increase in chloride content of 0.3 m.eq. per 100 g. body weight would produce an increase in osmotic pressure of about 3 per cent if no compensatory changes took place, assuming body water to be 70 per cent of the body weight and osmotic pressure to be equivalent to 300 m.osM./1. The osmoregulatory mechanisms are therefore probably sensitive to variations smaller than ±3 per cent of the osmotic pressure when such change occurs in 24 hours or less.

It was found that loss or gain of 150 to 238 μeq. Cl corresponded to a change in body water of one ml. However, the chloride concentration of the body fluid is lower than 150 m.eq./1, probably about 117 m.eq. as found by Robertson (1954) in the powan *Coregonus clupeoides*. Periods of steady uptake of chloride must consequently result in increasing concentrations of chloride and presumably also osmotic pressure of the body fluid. Periods of loss, on the other hand, must ultimately lead to decreasing chloride and osmotic concentrations. It thus seems as if the levels at which chloride and osmotic concentrations are maintained vary with the total chloride content of the body. It can further be assumed that the increase in chloride or osmotic concentrations which follows chloride uptake stimulates mechanisms that eventually result in net loss of chloride. Similarly, decreases in chloride or osmotic concentrations may be supposed finally to stimulate uptake and to reduce excretion of chloride, so that body chloride is once more increasing or perhaps kept constant for some time. Volume and osmotic regulation thus seem to be mutually dependent within a system that permits much larger variations in chloride content than in chloride concentration and consequently also osmotic pressure.

SUMMARY

Daily changes in chloride content and weight of undisturbed starving goldfish were followed during several weeks. Uptake or loss of chloride often continued for several days or even weeks at the time. Up to 83 and 89 per cent increases in chloride content were measured. Maximum daily gains were 17 per cent, and losses 22 per cent of total chloride content. A statistically significant correlation was found between daily chloride and weight changes. Retention or excretion of 150 to 238 μeq. Cl was followed by retention or excretion of one ml. water.

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RELATIVE EFFECTIVENESS OF DEHYDRATION AND NEUROHYPOPHYSIAL EXTRACTS IN ENHANCING WATER ABSORPTION IN TOADS AND FROGS

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It is well known that the vertebrate neurohypohysis contains substances which can increase the permeability to water of the skin of amphibians and reduce the rate of urine flow. The same effects can be produced by dehydration or by injection of hypertonic solutions. It has, therefore, been suggested that these treatments act by stimulating the neurohypophysis to liberate so-called water balance hormone. Ultimately, neurohypophysial activity should thus be responsible for at least part of the renal and cutaneous effects of hydropenia in amphibians (Eliassen and Jørgensen, 1951; Ewer, 1952; Jørgensen, 1950; Sawyer and Sawyer, 1952). It thus seemed of interest to compare the relative effectiveness of dehydration and neurohypophysial extracts in enhancing water uptake. Experiments were made on the toad *Bufo bufo* (L.) and the frog *Rana temporaria* (L.).

TECHNIQUE

Water uptake was measured as previously described (Jørgensen, 1949). The animals were placed in stainless steel wire cages and immersed in tap water. The rate of water absorption was determined by weighing animal plus cage at suitable intervals, generally one or one half hour. The animals were dehydrated in glass beakers covered by gauze, at a rate corresponding to a daily weight loss of about 10 per cent. Injections were given subcutaneously. Both mammalian and amphibian neurohypophysial extracts were used. Insipidin AB and Pitupartin AB served as mammalian preparations. Insipidin contains 20 international units of the pressor-antidiuretic principle per ml., Pitupartin 10 units of the oxytocic principle per ml. Insipidin has been found to be more effective than Pitupartin in enhancing water uptake in the toad. In the experiments on toads Insipidin was consequently used, 2–5 U being injected per 100 g. body weight to ensure maximum effect on water uptake (Jørgensen, 1950). In frogs the oxytocic principle is apparently more effective than the pressor-antidiuretic principle of commercial neurohypophysial preparations (Heller, 1945). In our experiments maximal rates of water uptake were induced by injection of about 5 U of Pitupartin per 100 g. frog. Extracts of the neurohypophyses of frogs and toads were made either in the usual way by extracting the minced glands for about 5 minutes with boiling 0.25 per cent acetic acid or by finely grinding the glands in a mortar with a little sand and a drop of Ringer solution. The comminuted tissue was suspended in Ringer solution and injected. Maximal stimulation of water uptake was obtained with amounts of extract corresponding to less than one-tenth of a gland from an animal of the same species and of the same size as that into which the extract was injected. In the present experiments extract from one-tenth of a toad gland was injected into each toad and one-fifth of a frog gland into each frog.

RESULTS

The effect of hydropenia on the ability of toads and frogs to take up water through the skin varies with the temperature, the season of the year and other factors which have not been investigated systematically. An example of the large seasonal variations that can be found in the effect of dehydration on the rate of water absorption is given in Figure 1. Water uptake of normally hydrated toads was 4 ml./hr./100 g. in June and 2 ml. in December and March at 23–24° C. In June a weight loss of some few per cent strongly increased the water uptake, whereas in December or March a weight loss of 8–10 per cent was needed in order to affect the permeability to water. The maximal rates of water absorption, too, have been observed to be higher in summer-toads than in winter-toads. Comparisons between the effect of dehydration and neurohypophysial extracts should therefore be made at the same time of the year. Table I collates the results of a number of such comparisons between the relative effectiveness of strong dehydration and neurohypophysial extracts in enhancing water absorption in toads and frogs.

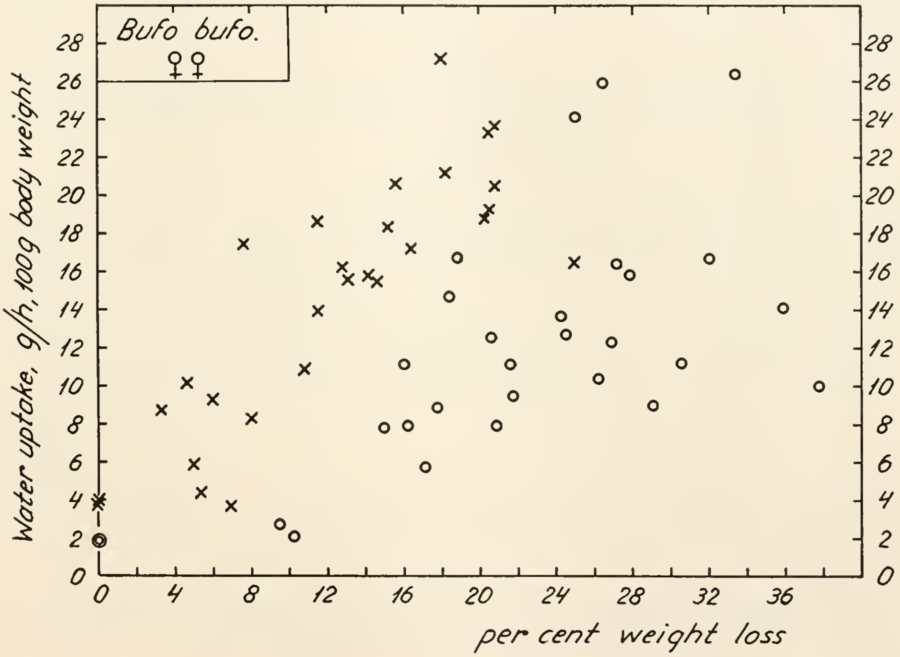

FIGURE 1. Water uptake in dehydrated female toads placed in tap water. × June; ⊙ December and March. Temp. 23–24° C.

In experiments 2 and 3 of the Table the same animals were subjected to both dehydration and to injections. In experiment 1 the two types of treatment were given to separate groups of toads that had, however, been treated uniformly up to the time of the experiments. Desiccation corresponding to 20–30 per cent weight loss was much more effective than injection of supramaximal doses of neurohypophysial extracts in all experiments made at the same time of the year. There was no difference between the effect of mammalian and amphibian extracts. The pituitary extracts, however, produced larger effects during summer than during winter. In May–June toads responded as strongly to the extracts as they did to dehydration in November–December. No difference was found in the responses of the two sexes.

The increase in sensitivity to water loss and neurohypophysial extracts coincides with the onset of sexual activity in the spring. In toads imported from Italy in February during the breeding period, 17–24 per cent weight loss resulted in water uptakes of 16–21 ml./hr./100 g. at 18–19° C. Extracts of 1/10 of a toad-neurohypophysis increased water absorption to the high values of 7–13 ml. At the same time Danish toads were still sexually inactive and showed the typical low "winter-response." The strong water balance reaction persists beyond the breeding period as may be seen from experiment 2 of the Table, performed one–two months after reproduction had taken place.

TABLE I

Effect of dehydration and injection of neurohypophysial extracts on the rate of water uptake in toads and frogs. Water uptakes are expressed in ml. per hour and 100 g. body weight. The figures represent means of 5–15 independent determinations. Ranges are given in brackets

No. of experiment	Species	Time of year	Temp. °C.	Sex	Normally hydrated	Dehydration corresponding to 20–30% weight loss	After administration of:		
							Ringer's solution	Insipidin 2–5 U/100 g.	1/10 toad neurohypophysis
1	*Bufo bufo*	Nov. Dec.	17–19	♀	1.1 (0.8–1.7)	7.5 (5.1– 9.5)	1.3 (0.8–2.3)	2.0 (1.1– 3.3)	1.9 (1.1–2.9)
2	*Bufo bufo*	May	21–22	♀	1.7 (1.1–3.5)	18.7 (12.9–34.8)	2.0 (1.5–2.9)	8.1 (5.3–10.7)	
		June		♂	1.8 (0.9–3.6)	20.9 (9.0–38.8)	2.3 (1.4–2.8)	7.4 (4.4–11.3)	
								Pitupartin 5 U/100 g.	1/5 frog neurohypophysis
3	*Rana temporaria*	May	23–25	♀ ♂	2.4 (1.6–3.9)	19.6 (16.3–22.1)		4.8 (2.3–6.5)	5.3 (2.5–7.8)

DISCUSSION

Dehydration was found always to be more effective than injection of neurohypophysial extracts in increasing the rate of water uptake through the skin of toads and frogs. If, therefore, enhanced water absorption after desiccation is produced by water balance hormone liberated from the neurohypophysis it follows that injected water balance principles must be less effective than hormone liberated from the animal's own neurohypophysis. However, in man and dog Pitressin can produce a maximally concentrated urine and thus be as effective as naturally liberated antidiuretic hormone (O'Connor, 1950; West, Traeger and Kaplan, 1954). More likely, therefore, the effect of hydropenia on the permeability of the skin of toads and frogs is not exclusively due to increased neurohypophysial activity. At strong dehydrations the neurohypophysis is presumably of only minor importance in enhancing the ability to absorb water.

SUMMARY

Comparisons have been made between the effect of strong dehydration (20–30 per cent weight loss) and of supramaximal doses of neurohypophysial extracts on the rate of water uptake through the skin of the toad *Bufo bufo* (L.) and the frog *Rana temporaria* (L.). Hydropenia increased the permeability to water much more than did injection of the pituitary extracts. Extracts of mammalian and amphibian glands were equally effective. The responses to desiccation and to injection of neurohypophysial extracts were larger during summer than during winter.

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ON PIGMENT CHANGES AND GROWTH IN THE BLUE-GREEN ALGA, PLECTONEMA NOSTOCORUM BORNET EX GOMONT

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Changes in pigmentation in the algae, as influenced by environmental conditions, have evoked considerable interest in the past and at present. It is the purpose of this paper to describe one of the lesser known instances in which a specific change in pigmentation follows a specific change in the environment of an alga, and some of the practical considerations that may be drawn therefrom.

The theory of chromatic adaptation in the algae commenced in the late 19th century with the observation that there was a relationship between color and depth of zone of growth of marine algae, and has had a controversial course since. Rabinowitch (1945) and Boresch (1932) may be consulted for an extended discussion. Several points have gradually become apparent.

One of the lesser publicized of these has been the observation that in certain blue-green algae, color may vary with nutrition in a dramatic fashion. Apparently the first such observation was made by Boresch (1910) with an unidentified member of the Oscillatoriaceae. He found that after a protracted period of culture, the alga lost its characteristic blue-green color and took on the light brown to golden color of a diatom. Experimentation showed that it was not the wave-length of light, but rather the depletion of nitrogen from the medium, and that alone, which was responsible for the color shift. In this and further work (Boresch, 1913; Magnus and Schindler, 1912; Schindler, 1913), all with members of the Oscillatoriaceae and especially species of *Oscillatoria* and *Phormidium,* it was determined that the color shift was due to a selective disappearance of chlorophyll and phycocyanin, thereby "unmasking" the carotenoids; that a yellowed culture could be made to return to green, sometimes within 24 hours, upon the addition of minute amounts of nitrogen [as little as "1/20,000 normal" KNO_3 (Boresch, 1910) (0.00425 grams per liter)] as nitrate or ammonium ions or various organic nitrogen sources; and that greening would take place in the absence of light. This work was done in the period of active debate concerning the validity of theories of chromatic adaptation. Light was indirectly involved in these experiments since the greater the availability of light, the sooner the nitrogen content of the medium would be used up, other factors being equal, and the quicker and more markedly the color shift would take place. It was noted that the chromatic adaptation, and color change due to nutrition concepts as related to light were ecologically antipathetic. The former states that color shifts occur to allow the organism to adapt itself for the better use of light received, thus increasing its phototrophic assimilation, while the latter states that phototrophic assimilation is decreased by the color shift because of the disappearance of the primary photosynthetic pigments. Although the green to yellow color shift reduces or eliminates phototrophic assimilation, it was held nevertheless beneficial to the alga in that it allows the organism to enter a state of dormancy until conditions ameliorate.

Material

In the course of other work a pure culture of a blue-green alga was obtained from a natural collection through the use of ultraviolet irradiation. This organism was identified by Dr. Francis Drouet as *Plectonema nostocorum* Bornet ex Gomont. It is somewhat unusual in its minute dimensions, being composed of sheathed trichomes of undifferentiated cells averaging 1.3 μ in width at the widest point by 1.8 μ in length. The sheath increased the width of the filament to 2.25–3.0 μ. Since no heterocysts, akinetes or other possibly resistant entities are formed by this species, and since it has no powers of morphological modification (except perhaps variation in width of the sheath), adaptive reactions to the environment are necessarily at the physiologic level.

Experimental

Nutritionally mediated color shift

Plectonema nostocorum had not been in culture long before the color shift was observed in some cultures. Cultures were grown in 250-ml. Erlenmeyer flasks in several liquid media. These were kept under conditions of controlled temperature (22° C. ± 1° C.) and light (40 foot candles, fluorescent source). Actively growing cultures are a brilliant emerald green. If they are kept in the same culture solution over an extended length of time in the light, the culture will, within the space of a few days, undergo a dramatic color shift from brilliant emerald green through yellow-green to a final yellow with slight overtones or orange or brown. In the latter condition, further lapse of time brings about a slow bleaching so that cultures that have been kept for many months assume the color and resemblance of dirty yellowish cotton-wool. Although at first it was assumed that this color shift was brought about by the death of the organism, further experimentation soon established the fact that it was, rather, based on the exhaustion of the mineral solution in which the organism was growing and that the organism even after many months in the new, yellowed condition was still viable. The addition of small amounts of nitrate to the yellow culture caused it to return to the original green at a very rapid rate. The beginnings of the color shift from yellow to green could be observed within about 24 hours and the shift became complete within three days. Several other elements added similarly had no such effect.

Spectrophotometric analyses of growing and dormant (yellow) cultures have been made. In preparation for this examination, living material was suspended in a few milliliters of culture solution and broken up to as small filament fragments as possible in a special small plastic mortar. The resulting suspension was diluted with distilled water to a degree necessary to give an optical density of approximately 0.65 at the wave-length of greatest absorption. The spectrophotometer used to measure the absorption at the various wave-lengths was a Coleman Universal, Model 14, following the procedures suggested by the manufacturer.

The spectral curve so obtained is somewhat flattened by turbidity and cell material other than pigments in the sample. It was not possible to break up the filaments entirely, and the resulting sample showed a range of particle size from just visible to the naked eye down to fragments of filaments one or two cells long. The measurements were checked at wave-length intervals of every 100 millimicrons for possible changes in the reading due to settling of the sample. It was found that the greatest effect was a change of only 0.01 units of optical density over the time of the entire assay.

Although no attempt has been made to determine the exact identity of the pigments contained in the cells of this alga, the absorption curve is reproduced (Fig. 1) together with the approximate maximum absorption values for the pigments known to occur in the Cyanophyta (Boresch, 1932; Cook, 1945; Strain, 1951). It will be seen that there is good correspondance between the maxima of pigments generally found in the Cyanophyta and the general shape of the absorption curve for this alga in the active state, except in the region around 640 and 650 millimicrons. Chlorophyll b and c-phycoerythrin are apparently absent in this organism.

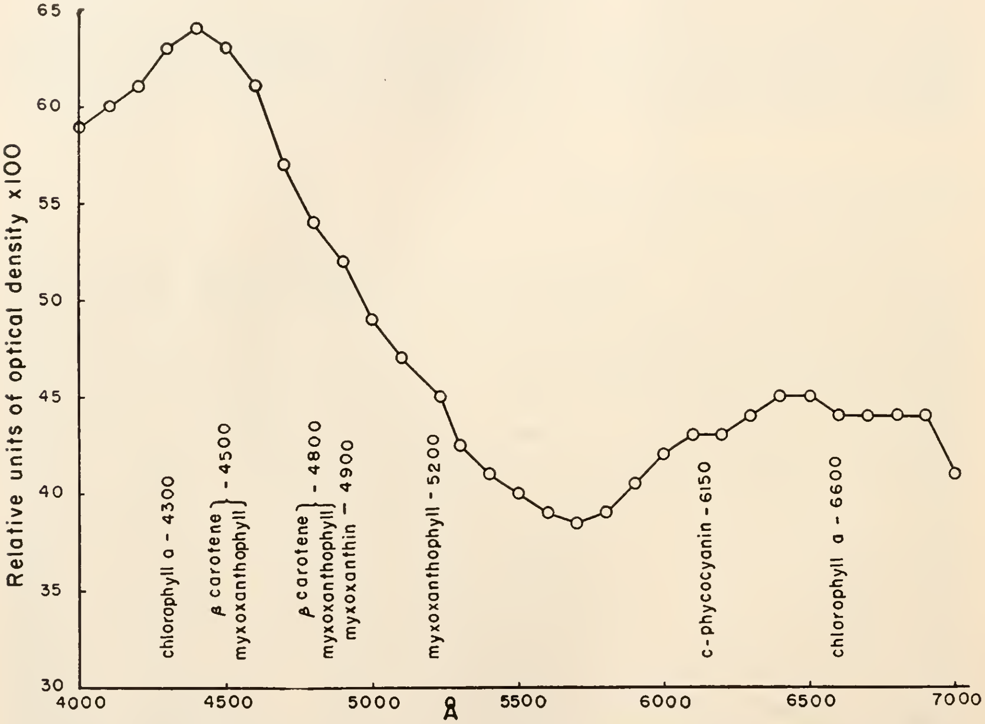

FIGURE 1. Spectrographic analysis of a green, growing culture of *Plectonema nostocorum.*

Spectrophotometric analyses of the living but yellowed pure cultures were made to determine what pigments had disappeared. As shown in Figure 2, both the chlorophyll and phycocyanin peaks are missing, the major absorption of the cultures now being represented by the presence of carotenoids only.

Growth estimation by pigment analysis

In further exploratory work with this organism it was necessary to develop an accurate method of measuring growth. Because of the small size, filamentous nature, and slow rate of growth of the organism, several of the more usual methods of measuring growth were found to be inadequate. Attention was then given to measurement of growth by means of pigment extraction and its quantitative determination.

Growth is an elusive concept and while it is recognized that pigment measurements do not necessarily precisely indicate the amount of organic matter fixed in the organism because of superimposed ecological variations, on the other hand, if one allows a more dynamic concept of growth such as total current organic matter-producing potential, then pigment measurement assumes a greater significance (Harvey, 1950). Careful pigment analysis can also be used to characterize and estimate plankton populations (Richards, 1952).

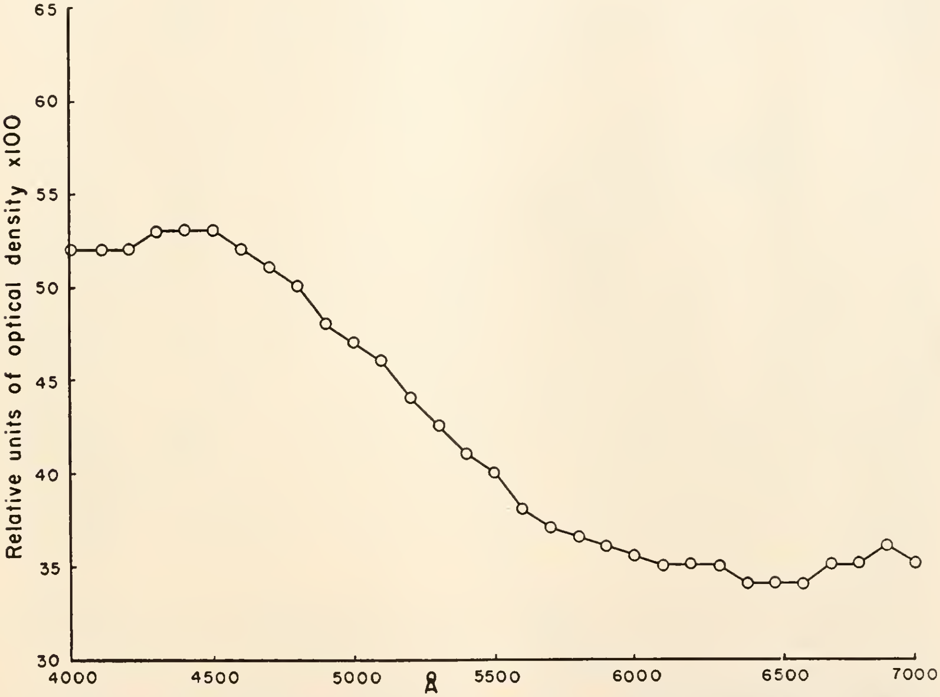

FIGURE 2. Spectrographic analysis of a yellowed culture of *Plectonema nostocorum*.

The first to use a quantitative pigment measurement for determination of growth was Harvey (1934) who extracted (with 80% acetone) the pigment from marine plankton collected by net from a known volume of water, and compared this extract visually with calibrated dilutions of a colored standard. This method proved satisfactory for Harvey's work which was with natural populations consisting largely of diatoms. Later Riley (1938) attempted to apply the method to the measurement of fresh water plankton populations but found that these had a too great and too variable carotenoid content. He was able to adapt the method to his needs by first extracting the chlorophyll from the total pigment complement and measuring it alone visually against calibrated standards. Gardiner (1943) determined, however, through microscopic examination of extracted sample residue, that under these conditions, not all species give up their pigments to the solvent. Further critical work was done by Tucker (1949) who compared results as determined by Harvey's method with actual counts. He found that the correlation in natural populations was not close, and it was his conclusion that it is impossible to get a fixed plankton count as equal to a particular pigment density. This is corroborated in more recent work by Berardi and Tonolli (1953) and Ramamurthy (1953). This might have been expected because of the diverse nature of plankton, containing, as it does, representatives of a wide range of plant groups and an even wider range of animal life, its relative composition depending upon numerous factors such as season, latitude, mineral composition of the waters and others. Comparisons of pigment analyses after Harvey with dry weight determinations by Margalef (1954) have also given a poor correlation even when uni-algal cultures were used. Variation in dry weight per one Harvey and correlated unit of pigment was greater than one power of ten with variations in several factors of culture.

Since none of the above listed difficulties ensues if pigment extractions are made from a pure culture of a pigment-producing organism which has been cultivated under carefully controlled conditions, it might be assumed that the amount of pigment so extracted, measured photoelectrically, would correlate closely with the amount of growth as measured by any other reliable method, especially if the chosen pigment was closely associated with the basic physiology of the cell as are the photosynthetic pigments in autotrophic organisms. This has been borne out in at least one instance. Tucker (1949) found that the coefficient of correlation between the number of cells per unit volume in a pure culture of *Ankistrodesmus,* as measured at various times during the course of its growth, and the amount of chlorophyll per unit volume as sampled at the same times, was 9.97 which represents very close agreement.

On the basis of the above information an attempt was made to establish a workable method of determining growth of the alga through the use of photoelectric measurements of pigment extractions. The immediate problem developing was the decision of which pigment to extract and measure or whether it would be more desirable to measure all pigments at once. Because, as previously described, when the nitrogen composition of the medium becomes sufficiently reduced, the alga loses its green pigments and phycobilins and turns yellow, any method to be fully satisfactory should allow the quantity of growth to be accurately measured whether the culture is in the yellow or green condition. Thus extraction must be done in such a way as to obtain only the yellow pigments which are always present during the growth of the alga and remain after growth ceases.

Extractions of cultures in both the green and yellow conditions were made with various solvents such as acetone, petroleum ether, and methyl alcohol and the extracts were analyzed for pigment complement with the Coleman spectrophotometer as previously described. The effect of the use of heat to destroy enzyme activity quickly and thus prevent degenerative pigment changes was also investigated as opposed to extractions without this previous treatment. It was found that extractions of green cultures without heat and with 80% aqueous acetone yielded the same pigments as were obtained from yellow cultures treated similarly. The phycobilins are water-soluble and are not extracted from the cells by this method. On the other hand the chlorophyll is apparently destroyed in the extraction. It is quite apparent to the eye that as the acetone is added to the culture, there is a definite and rapid yellowing both of the solution and the filaments themselves. Although acetone solution is a normal procedure for the successful extraction of chlorophyll, it is probable that in this case the chlorophyll is destroyed by reason of the pH at which the extraction is done. Chlorophyll is stable only near neutrality or at a slightly acid reaction. Extraction in this instance is from a culture which has been grown at a pH of 10. Although the culture solution is removed before extraction, the filaments presumedly remain basic. In any case, that the chlorophyll is destroyed and does not appear in the solvent may be seen in the spectrographic photometric analysis of the latter. Figure 3 represents such an analysis in which the curves for 80% acetone extractions of both green and yellow cultures are

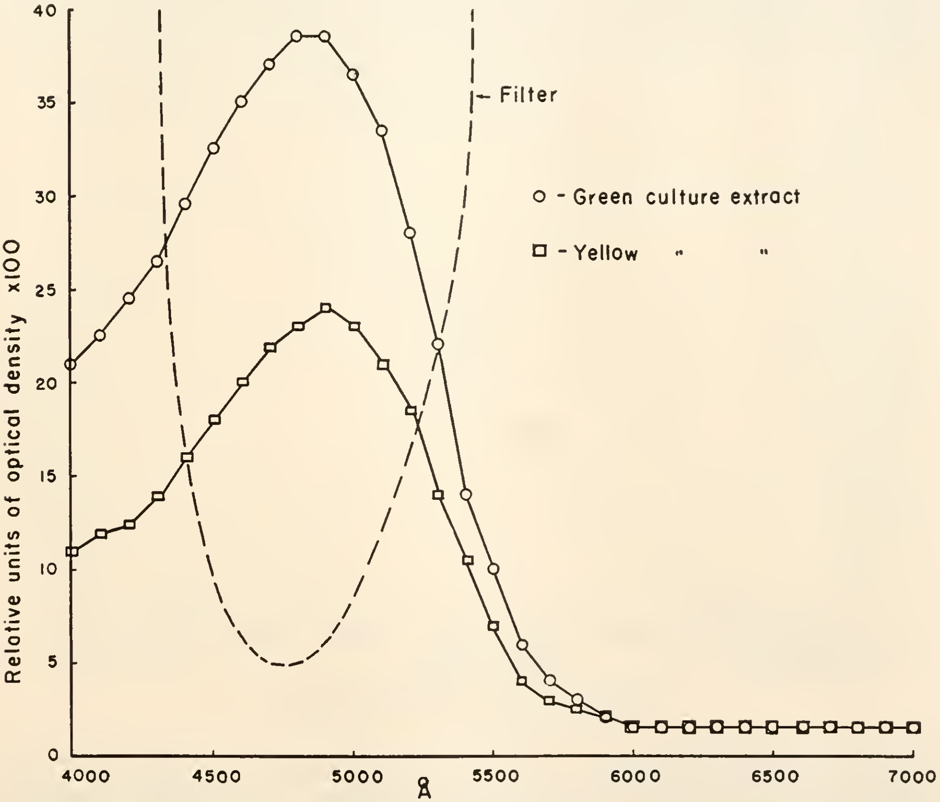

FIGURE 3. Spectrographic analysis of acetone extracts of green and yellow cultures of *Plectonema nostocorum.*

plotted. It will be seen that the absorption curves of these are similar, and it is presumed that the pigments are the same. The fact that the absolute amount of pigment is different is not significant since not only were the cultures grown under different conditions for different lengths of time, but also different dilution factors were used in their preparation for analysis.

It should be possible, therefore, to determine the amount of growth in a culture, whether the latter be yellow or green, through a photoelectric determination of the amount of pigment contained in an 80% acetone extract of the culture, other conditions being controlled and equal. A filter was obtained, for use in the Klett-Summerson colorimeter, whose transmittance characteristics resembled the absorption curve of the pigment extract. The transmittance curve for the filter, Wrattan (Eastman Kodak Company) gelatin filter number 45, is reproduced in Figure 3.

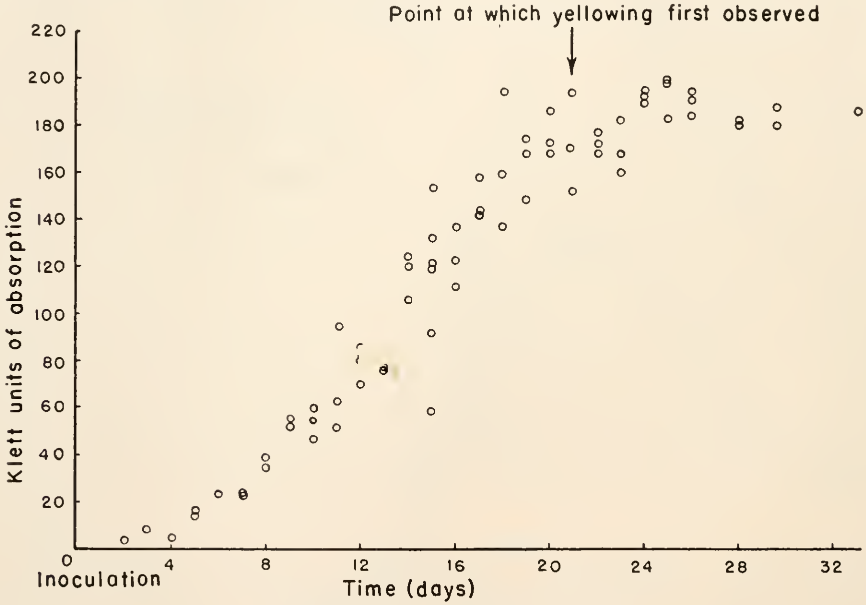

FIGURE 4. Plot of raw growth data.

Three of these filters in series equaled the transmittance of a glass filter made expressly for use in the Klett-Summerson instrument. It was further determined that the heat developed at the surface of the filter was not great enough to damage the gelatin, at least over the length of a normal pigment analysis.

A series of 250-ml. Erlenmeyer flasks was prepared. The nutrient solution was made up with CP grade reagents according to Chu's No. 10 formula (Chu, 1942) in glass-redistilled water and Chu's micrometabolic elements (Chu, 1942) made up similarly were added. The pH was adjusted to 9.7. One hundred ml. of nutrient solution were placed in each of the flasks and autoclaved. Inoculation of all flasks was from the same culture at the same time. One milliliter of this culture, which had been homogenized as much as possible in the Waring Blendor, and which had previously been tested for purity from contamination by inoculation into meat extract broth, was aseptically introduced into each of the flasks. These were then incubated under constant conditions of light and temperature.

Starting two days from the date of inoculation, one or more cultures were removed daily and assayed in the following manner. The culture solution was filtered through a No. 1, 4.25-cm. Whatman filter disc. The filter paper was then placed in the flask, and the algal material was extracted with 80% aqueous acetone for one hour. The extract was then filtered into a test tube and compared with an 80% acetone blank for pigment absorption using the filter described above in the Klett-Summerson instrument.

The raw data are plotted in Figure 4. It should be noted that the number of cultures removed per day varied from one to six. It should also be noted that the units of absorption are arbitrary. The scale of the instrument is plotted directly in a logarithmic fashion; thus the figures are directly proportional to the concentration of the pigment being read (Lambert-Beer Law). A calibration factor, determined by reference to a known standard, may be introduced so that the final readings may be expressed in units of defined value. This was not done. Precautions were taken to insure the flasks being assayed at random and to prevent any unconscious selection on the basis of color.

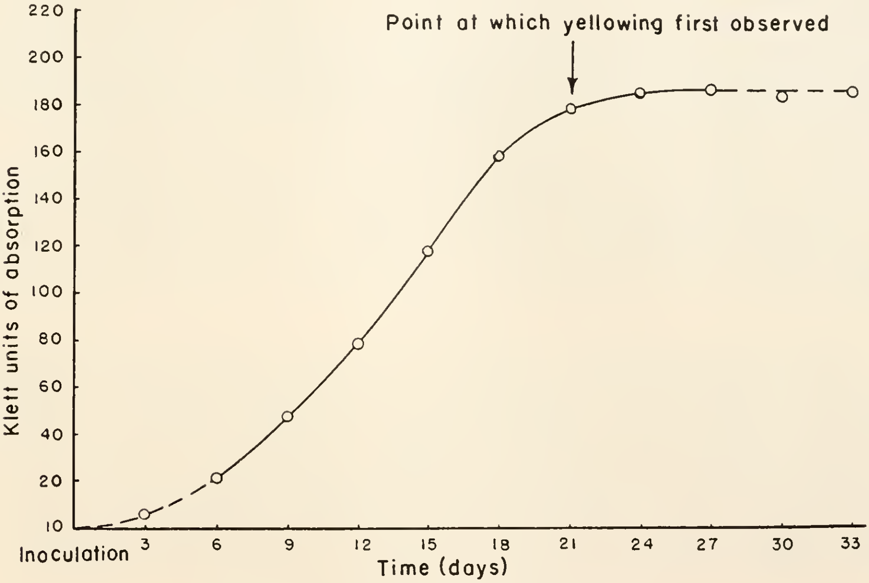

FIGURE 5. Plot of averaged growth data.

The following discussion is based upon the curve reproduced in Figure 5. This curve was prepared by grouping the raw data in groups of three days, each day being given equal value, and plotting the mean of the resulting groups. It must be noted that different numbers of cultures were assayed on different days, so that there is not the same degree of statistical reliability throughout the entire curve. The greatest reliability attaches to the central part of the curve.

The point at which yellowing was first observed in the cultures by eye is noted in Figure 5. It will be seen that there is no drop in the curve at this point. It can be expected that there will be a slow decay in the curve from this point, or shortly thereafter, onward through the gradual depauperation of the culture.

As has been stated, it has proven impossible to relate the figures obtained by this method with any other criterion of growth attempted. Dry weight determinations of parallel cultures were so small as to always fall within the limits of possible experimental error until the cultures were half grown. This argues for the sensitivity of the pigment method.

Discussion

Since both chlorophyll and phycocyanin molecules contain considerable amounts of nitrogen, and since the presence or absence of nitrates from the culture medium mediates color shift, it is concluded that the alga is able, when nitrogen becomes unavailable to it from the medium, not only to cease active repairing and construction of nitrogen-containing pigments, but to remove nitrogen actively from already constituted pigment molecules, a conclusion based on the very short time in which the color shift from green to yellow takes place.

Because of the rapidity with which greening takes place in this organism, it is possible to effectively separate pigment synthesis from other growth phenomena.

In this study, pigment evaluation was used as an index of growth. Despite the considerable experimental evidence of others to the inadequacy of this procedure under different conditions, the method appears to have been successful in this instance.

Summary

1. Spectrographic analyses of cultures of *Plectonema nostocorum* are reported, which demonstrate that the nitrogen-containing pigments disappear in the living alga when nitrogen becomes depleted in the culture medium.

2. Re-greening of cultures upon the addition of nitrogen is rapid and can serve to separate the process of pigment synthesis from other processes of growth.

3. Extraction and measurement of the carotenoid pigments at any stage in growth under controlled conditions is considered a reliable index of growth.

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SKELETON FORMATION OF SEA URCHIN LARVAE. I. EFFECT OF CA CONCENTRATION OF THE MEDIUM[1]

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Although many reports are available concerning the morphological development of the sea urchin skeleton (v. Ubisch, 1913; MacBride, 1914; Mortensen, 1921, 1931; Goldon, 1926a, 1926b, 1928; Onoda, 1931, etc.), the underlying mechanism of its formation is an aspect which has not been covered by previous workers.

In marine molluscs, on the other hand, it is known that the calcium utilized in the formation of the shell is derived in part from the sea water in which the animals live. Galtsoff (1934) reported that the amount of calcium deposited to form the oyster shell was many times greater than that which could be stored in the tissues. Orton (1925) noticed in the oyster that increase in the size of the shell took place even in the absence of food. Fox and Coe (1943), in their studies on *Mytilus*, showed that the amount of calcium obtained from organic food alone was not sufficient to account for the amount deposited in the shell. In recent studies, it has been proved that an isolated "mantle-shell preparation" is able to deposit calcium from the sea water onto the shell (Hirata, 1953; Jodrey, 1953). Bevelander and Benzer (1948) have shown that there is a corresponding reduction in the calcium of the regenerating molluscan shell (*Pedalion alatum*) when the calcium content of the sea water is reduced. Further, Bevelander (1952) agrees with Robertson (1941) in the conclusion reached in Robertson's comprehensive review dealing with calcification in molluscs, that molluscs can utilize calcium in the ionic form. This conclusion is further corroborated by Rao and Goldberg (1954), who cultured *Mytilus* in filtered sea water.

In sea urchin larvae, a direct dependence on sea water for the salts or ions used for the larval skeleton is shown by the following facts: the beginning of spicule formation precedes the completion of gastrulation, hence takes place before feeding starts; spicules continue to grow in cultures which are without food; exogastrulae having no functional digestive tract are able to form typical skeletons like those of normal plutei (Okazaki, 1956). In earlier studies, it has been stated that spicules do not develop in sea water in which the calcium is seriously reduced (Pouchet and Chabry, 1889) and also that the absence of magnesium and sulphate ions arrests the spicule formation of sea urchin larvae (Herbst, 1904). However, hardly any details about skeleton formation have been clarified so far.

In the present paper, the effects of the Ca concentration of the culture medium on skeletal development will be reported as a preliminary to the study of the mechanism of skeleton formation.

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[1] This research was supported in part by the Ministry of Education Research Expenditure.

Materials and Methods

The larvae of *Hemicentrotus pulcherrimus* were principally used, because the form of the skeleton of this species is the simplest to be found among the sea urchin larvae available at Misaki. As supplemental materials, *Pseudocentrotus depressus, Clypeaster japonicus* and *Mespilia globulus* were used.

Ca-low media were prepared by mixing Ca-free sea water [2] and natural sea water in 9 : 1, 8 : 2, 6 : 4, 4 : 6 and 2 : 8 ratios. As Ca-high media, 0.36 *M* $CaCl_2$ was mixed with natural sea water in 11 : 629, 11 : 309, 11 : 149, 11 : 69 and 11 : 29 ratios. The resultant Ca concentrations of the whole series of mixtures correspond to about 0.1 ×, 0.2 ×, 0.4 ×, 0.6 ×, 0.8 ×, 1.5 ×, 2.0 ×, 3.5 ×, 6.0 × and 10.0 × the calcium content of natural sea water. For convenience, these media will be referred to as 0.1 Ca, 0.2 Ca, 0.4 Ca . . . , respectively.

Larvae were cultured in Syracuse watch glasses containing about 15 cc. of medium. In these cultures, the larvae must be placed in a single layer, so as to minimize variations in the rate of development which would lead to individual differences in skeletal size. As long as the number of larvae in a container is kept below such a level, further reduction in the larval number per container does not affect the skeleton formation. This latter situation indicates that it is the concentration of calcium and not the absolute quantity of calcium available per larva which should be taken into consideration.

In all the experiments reported in this paper, the larvae were cultured in the absence of food.

Experimental Results

Before describing the effects of media with modified Ca concentrations on skeleton formation, it seems appropriate to mention their effects on the general developmental process of sea urchin larvae. These are summarized in the following five points.

(1) *Time of hatching.* In Ca-low media, no retardation of development was obvious in reference to the hatching time, while in Ca-high media, the time of hatching was more or less retarded. However, this retardation of hatching time in Ca-high media is not simply due to a generally delayed rate of development, judging from the time of mesenchyme formation and gastrular invagination, as described below.

(2) *Time of beginning of mesenchyme immigration and gastrular invagination.* With reference to the time of the onset of these activities, larvae in 0.1–3.5 Ca did not show any difference from the control, except occasional early beginning in 0.1 and 0.2 Ca. In 6.0 and 10.0 Ca larvae, however, both activities were retarded in comparison with the controls.

(3) *Gastrulation.* In 0.2–6.0 Ca, gastrulation proceeded in an almost normal manner. Larvae cultured in extreme concentrations (0.1 and 10.0 Ca) frequently developed into exogastrulae, as described in previous papers (Dan and Okazaki, 1956; Okazaki, 1956).

(4) *Size of the blastocoel.* In Ca-low media, the size of the larval blastocoel

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[2] NaCl 26.5 g., KCl 0.7 g., $MgSO_4 \cdot 7H_2O$ 11.9 g., $NaHCO_3$ 0.5 g. plus distilled water to 1000 cc.

became larger than that of the control larvae. This was so particularly in 0.8 and 0.6 Ca. In Ca-high media, on the other hand, the blastocoels became smaller in proportion to the concentration of Ca present, in the same manner as was reported by Dan (1952) in this species.

(5) *Formation of coelom.* In the extreme concentrations, the secondary mesenchyme cells failed to form a typical coelom. They either formed a single vesicle on the tip of the archenteron or dispersed without coordination.

From the foregoing facts, it can be said that the concentration range of Ca which allows essentially normal development is from 0.2 to 3.5 times that of sea water.

I. Limiting concentration of calcium necessary for skeleton formation

In larvae which were cultured in even as low a concentration as 0.1 Ca from the two- or four-cell stage, doubly refractive skeletons were formed, although their form was atypical and the time of their formation was much delayed, as will be described below. If larvae reared in sea water were transferred to Ca-low sea water after the formation of tri-radiate spicules, skeletal growth continued for a short time even in 0.05 Ca, in which the general development was ultimately impaired. These facts show that the Ca threshold for skeleton formation is lower than that for harmonic development of the whole larva. Such being the case, the minimal calcium concentration required for skeleton formation itself is impossible to decide.

TABLE I

Comparison of the time of the beginning of the skeleton formation in various Ca concentrations. Numbers indicate the ratios of hours required between insemination and skeleton formation in experimental media over the control. Material: Hemicentrotus pulcherrimus

Stage of transfer \ Ca concentration	0.1	0.2	0.4	0.6	0.8	S.W.	1.5	2.0	3.5	6.0	10.0
4-cell						1.00	0.99	1.00	1.00	1.04	1.08
32-cell	2.22	1.69	1.31	1.03	1.00	1.00	1.00	1.00	1.00	1.05	1.07
Morula	2.80	1.87	1.17	1.05	1.01	1.00	1.00	0.99	1.00	1.05	1.07
Blastula before hatching	2.65	1.71	1.15	1.02	1.00	1.00	0.99	0.99	1.00	1.04	1.13
Swimming blastula with no mesenchyme cells	2.20	1.69	1.29	1.03	1.00	1.00					

Bevelander and Benzer (1948), in their paper dealing with shell regeneration in young specimens of *Pedalion,* reported that when an operated animal was placed in sea water containing ⅛ of the normal calcium content, the organic matrix was completely devoid of calcium, although the matrix was formed in a normal manner. For sea urchin larvae, however, a medium having such an effect cannot be prepared by calcium reduction alone.

It is frequently the case that in the larvae of *Clypeaster japonicus* and *Mespilia globulus* cultured in 0.1 Ca, skeletons as such failed to appear; instead, minute doubly refractive granules were formed in the mesenchyme cells.

II. Time of the beginning of skeleton formation

Larvae fertilized and cultured in sea water were transferred to media of various calcium concentrations at various stages before the appearance of the mesenchyme

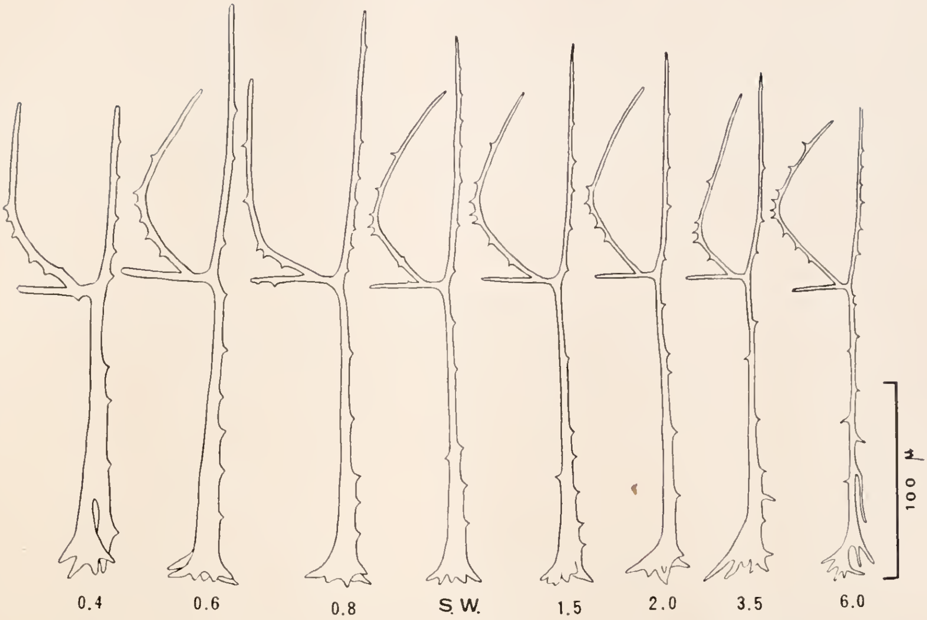
0.4
0.6
0.8
S.W.
1.5
2.0
3.5
6.0
100 μ

Figure 1. Skeletons of *H. pulcherrimus* larvae reared in media with varied Ca concentrations from 0.4 to 6.0 times that of sea water.

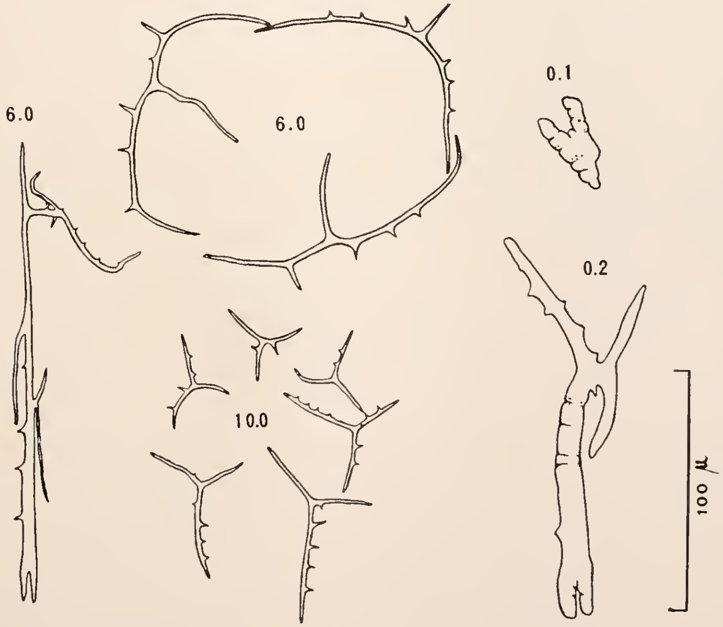
6.0
6.0
0.1
0.2
10.0
100 μ

Figure 2. Skeletons of *H. pulcherrimus* larvae in the extreme Ca concentrations (0.1, 0.2, 6.0 and 10.0 × normal sea water.) Larvae were transferred to such media before skeleton formation.

cells, and the times of the beginning of skeleton formation were compared. As the initial stage of skeleton formation, the time was recorded when doubly refractive granules were recognized by a polarization microscope in 50% of the larvae in a given culture. The results are summarized in Table I.

As far as pre-mesenchyme blastulae were concerned, the stage at which the larvae were transferred did not affect the results. Generally speaking, in Ca-low media, the time of the beginning of skeleton formation became later in proportion to the dilution of Ca, while the increase in Ca content had only a slight effect in this sense. In considering the delay in skeleton formation at extreme concentrations, the retardation of the developmental rate must also be taken into account.

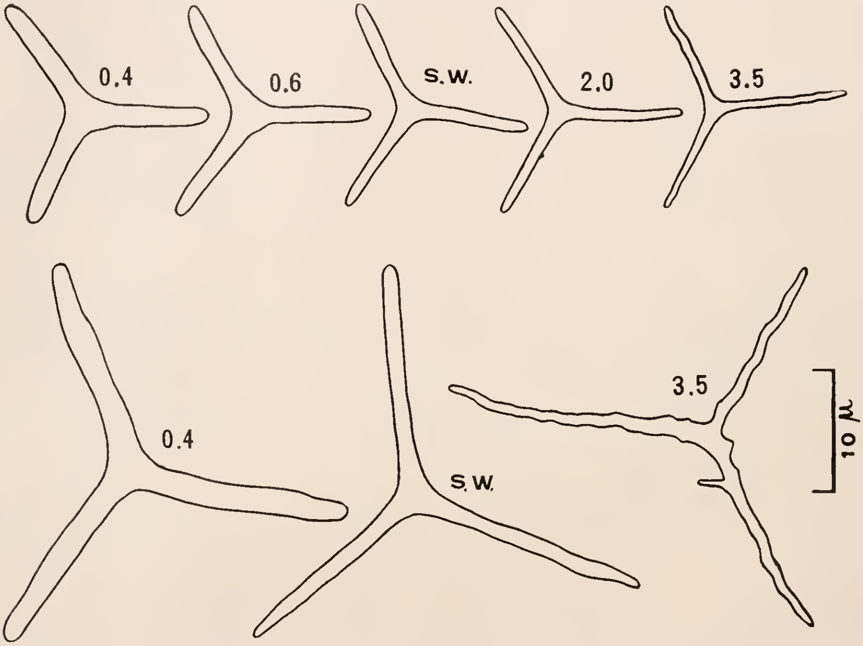

FIGURE 3. Camera lucida drawings of spicules in media of various Ca concentrations at two stages of skeletal development. Note correlation between Ca decrease and thickening of spicules. Larvae transferred to experimental media from sea water at gastrula stage with rudimental spicule granules.

III. Form of the skeleton in calcium-low and -high media

When larvae were cultured in media with various Ca concentrations from a stage before the beginning of skeleton formation, the forms of the skeletons of these larvae at "the final stage"[3] showed characteristic features according to the calcium content, as is illustrated in Figures 1 and 2.

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[3] By "the final stage" is meant the stage in which the larval skeleton reaches its maximum of development in foodless culture. Control larvae at this stage have four long arms, a func-

On the whole, in the range of 0.4–6.0 Ca, the skeletal forms of the experimental plutei were roughly similar to those of the controls (Fig. 1). If they are compared in detail, however, a slight shift can be recognized in the thickness and length of the skeleton, and in the shape and number of hooks, paralleling the concentration shifts in calcium. The thickening of the skeleton was inhibited in proportion to the increase in the Ca content of the medium; this was noticeable even as early as the tri-radiate spicule stage, as is indicated in Figure 3. The elongation of the skeleton was inhibited in proportion to the decrease in Ca concentration within the range 0.1–0.4 Ca (see Figs. 1, 2). There was a corresponding reduction in the number and the sharpness of the hooks, if the calcium content of the medium was reduced, and finally in 0.1 Ca, the hooks were missing.

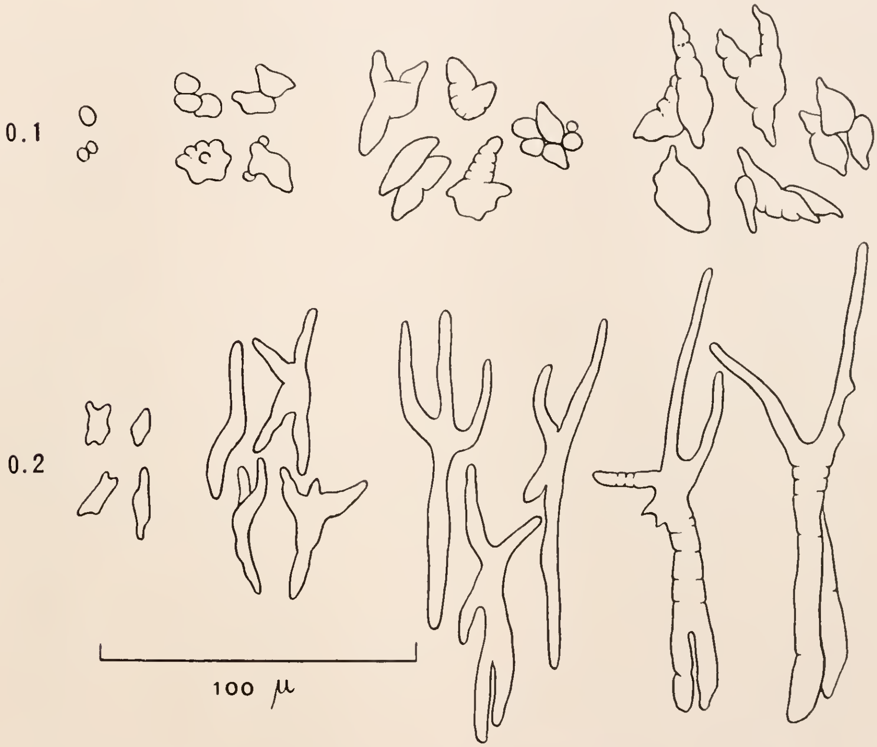

FIGURE 4. Process of skeletal growth in *H. pulcherrimus* larvae in Ca-low media (0.1, 0.2 × Ca).

At extreme concentrations outside the above range, there occur distinct differ-

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tional digestive tract and a pair of coeloms. Since, however, the destined length of life of the experimentals turns out to be as long as that of the controls or even longer in some solutions, the data of the controls and experimentals are roughly comparable. When experimental larvae died before the end of the experiment, they were omitted from the data.

ences, with respect to the form and developmental process, between the skeletons of controls and of experimentals, as will be described below.

Skeletons in media extremely low in calcium. As was mentioned above, doubly refractive skeletons could be formed in larvae reared in such Ca-low media as 0.1 or 0.2 Ca from a stage before skeleton formation. In such media, these doubly refractive skeletal rudiments never developed into the tri-radiate form found in the controls. However, the rudiments continued to grow in size as irregular masses (Fig. 4). As a natural consequence, the skeletons at the final stage of such Ca-low larvae were extremely atypical.

On the other hand, if the larvae were transferred to 0.1 or 0.2 Ca after the formation of tri-radiate spicules, new skeletal development in the Ca-low media was recognized as thickenings at each of the three spicule tips, globular in 0.1 Ca and ovoid in 0.2 Ca. (This fact may indicate that in the normal process of skeleton formation, Ca deposition is more vigorous at the tips than at the other points of the skeleton.) Subsequently, all parts of the spicules gradually thickened until skeletons at the final stage resembled typical pluteus skeleton, although they were much dwarfed (Fig. 5).

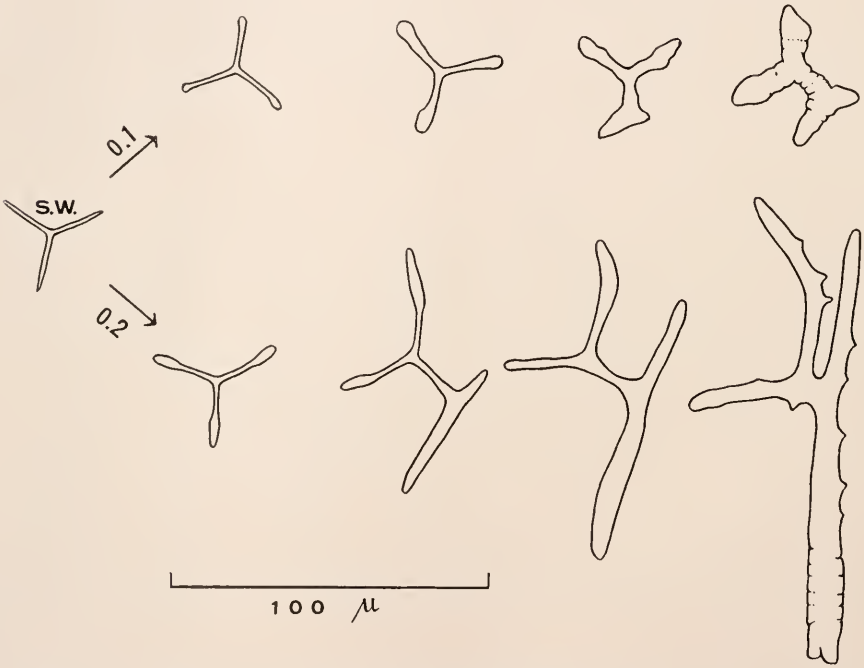

FIGURE 5. Modification of shape of tri-radiate spicules on transfer to Ca-low media (0.1 and 0.2 Ca).

If the larvae were transferred to Ca-low media after the pluteus stage, the thickening of the skeleton was found first at all tips and hooks of the skeletons (Fig. 6). In all cases in the two media lowest in Ca, it was a conspicuous feature of skeletal growth that the proportion of length to thickness was very small, par-

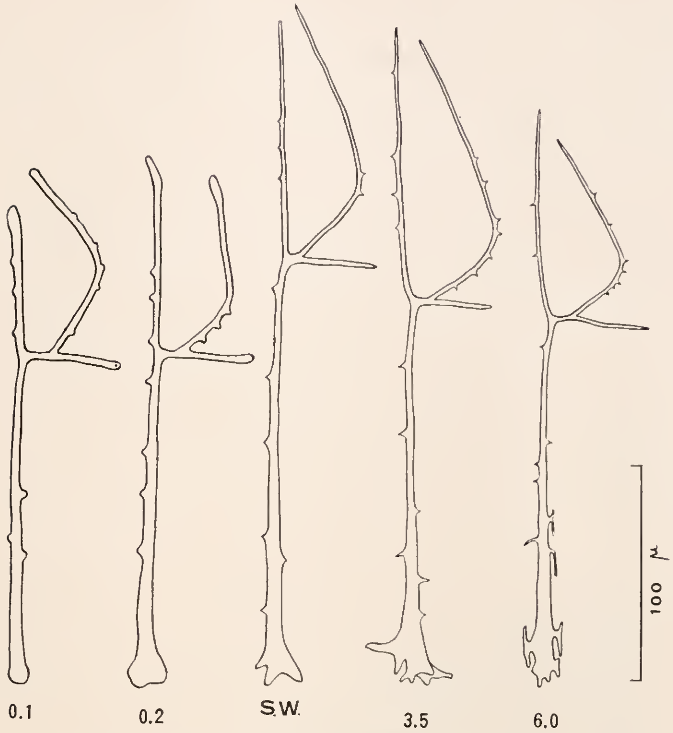

FIGURE 6. Effect of late transfer of *H. pulcherrimus* plutei to experimental media. Note effects of Ca change on form of hooks and skeletal tips.

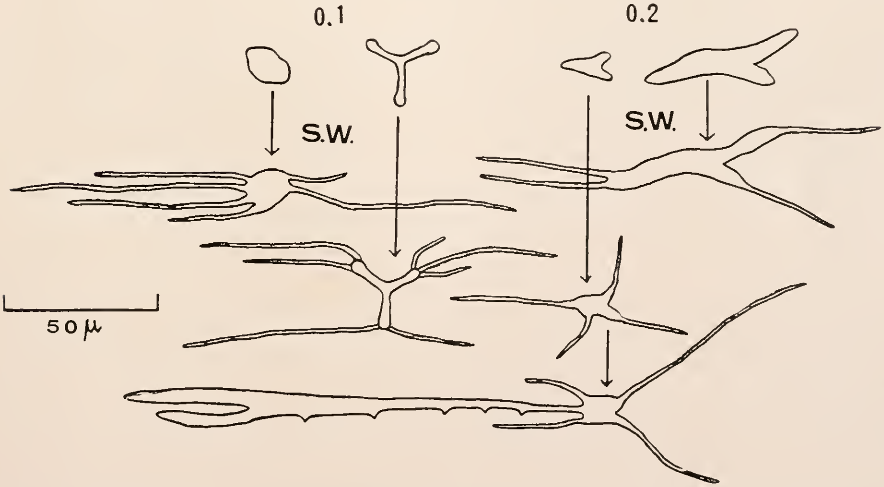

FIGURE 7. Skeletons of *H. pulcherrimus* larvae transferred back to sea water from Ca-low media (0.1 or 0.2 Ca), showing terminal elongation of slender skeletal rods.

ticularly in 0.1 Ca, and the surface of the skeleton became uneven as it thickened, as is shown in Figures 2, 4 and 5.

As a converse experiment to the above cases, if larvae which had been in 0.1 or 0.2 Ca were returned to sea water during the course of skeleton formation, several spindly processes invariably grew out from the edge of the massive skeleton, without regard to the stage of transfer (Fig. 7). In this case, if the larvae were transferred to 0.4, 0.8 or 1.0 Ca (sea water), the shape of the parts newly formed on the old one became longer and thinner in proportion to the new concentration of Ca.

Skeletons in extremely high calcium media. If the larvae were cultured in 10.0 Ca from a stage before skeleton formation, several spicule centers were formed, each of which developed into a tri-radiate spicule. These rarely attained a sufficient size to serve as functional pluteus skeletal parts (Fig. 2). Such abnormality was sometimes obtained in 6.0 Ca larvae. However, if sea water larvae were transferred to 6.0 or 10.0 Ca after the formation of tri-radiate spicules, excess centers of the spicules did not form and new skeletal development was continued to form the typical pluteus skeleton on the basis of the old spicules. In either case, the usual thickening failed to take place in such skeletons and there was also some reduction in elongation.

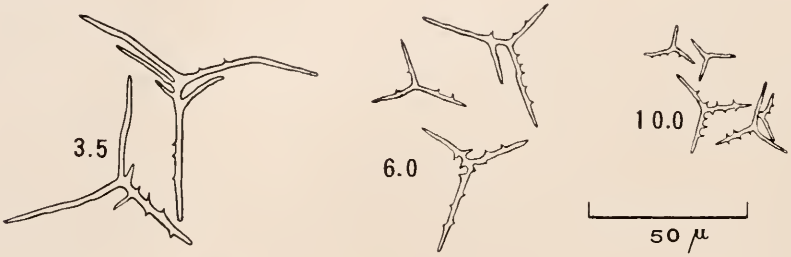

FIGURE 8. Spicules of *Hemicentrotus* gastrulae having precocious hooks in Ca-high media.

In 6.0 or 10.0 Ca, the side hooks of the skeleton which normally appear at the pluteus stage were formed prematurely at the gastrula stage, *i.e.*, at the tri-radiate spicule stage (Fig. 8). Such precocious development of the side hooks was also frequently observed in 3.5 Ca. If the transfer to Ca-high media was done after the pluteus stage, the shape of the hooks became sharper and longer as the Ca content of the medium was increased (Fig. 6).

IV. The effect of Ca concentration on apparent increase in length of the skeleton

Putting aside the aberrant skeletons formed in the two extremes of the Ca range, a comparison of the skeletal growth by microscopical measurement was made within the range of 0.4–3.5 Ca. Since the skeletal growth of individual larvae was very variable even among control larvae derived from the same batch, and further, since these individual differences became increasingly greater with the increasing divergence of the Ca concentration from that of sea water, particularly on the dilute side, the data given are averages of specimens showing good development (see Glaser, 1950).

To obtain uniform initial length of the spicules, the larvae were allowed to develop in sea water until the formation of the tri-radiate spicules had taken place, before they were transferred into media of the above range of calcium, and the lengths of the spicules in the various media were compared at intervals thereafter. In spite of variation in the details of the apparent skeletal growth, the general trend showed that a high concentration of calcium caused better skeletal growth

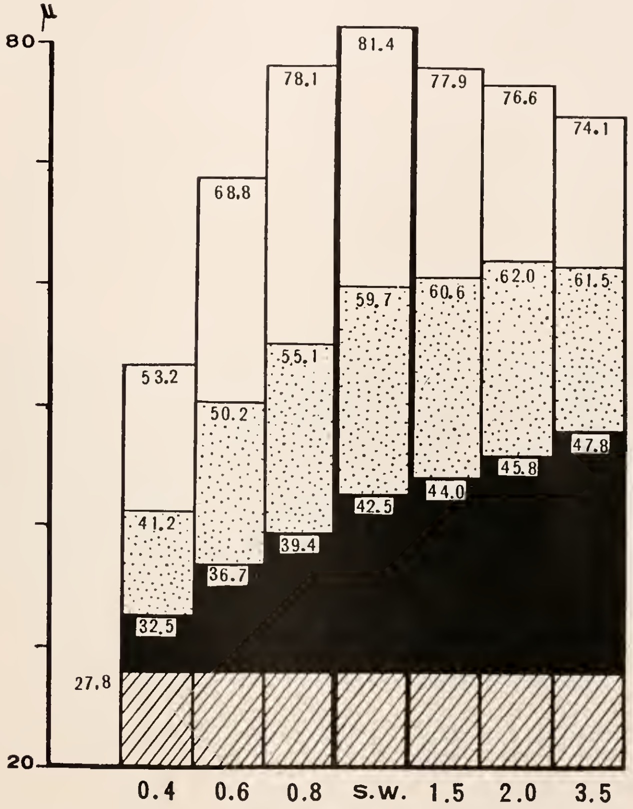

FIGURE 9. Apparent skeletal growth following transfer from sea water to media with various Ca concentrations. Material: *Hemicentrotus pulcherrimus* gastrulae. Ordinate: actual lengths of spicules calculated from camera lucida drawings. Abscissa: Ca concentrations of media. Columns with shading indicate initial length of spicules at transfer. Black, dotted and white columns indicate increase in skeletal length during each two hours after transfer at 16.5° C.

than sea water for a short time following transfer, but became harmful later on, while low calcium was relatively harmless.

The data of one experiment presented in Figure 9 illustrate this tendency. In this case, the length of the skeleton reached during the first two-hour period after transfer increased corresponding to the Ca concentration.[4] For the second two-hour period, however, the growth rate of the skeleton in the Ca-high media came down to approximately the same level as that of sea water, and for the third two-hour period, the increase in skeletal length in Ca-high media became inferior to that in sea water. On the other hand, the growth rate in Ca-low media decreased in proportion to the decrease in Ca concentration at first, but improved later.[5]

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[4] Maximum increase in skeletal length during the first period was found sometimes in 1.5 Ca or in 2.0 Ca or in 3.5 Ca. In all cases, however, the skeletons formed in Ca-high media were longer than those in sea water.

If the total lengths of post-oral rod + body rod at the final stage in various media were compared, the skeletons in 0.6 or 0.8 Ca were usually longer and in Ca-high media were shorter than the skeletons in sea water.

V. Spatial relations between ectoderm and skeleton

At the time when tri-radiate spicules were formed in sea water, spicules had not yet been formed in 0.1 or 0.2 Ca, but the ectoderm at the position of the presumptive post-oral arms was wrinkled (Fig. 10). If such larvae were transferred to sea water, the wrinkled ectoderm gradually expanded and formed the arm, accompanied by development of the spicules. However, if the larvae were kept in 0.1 Ca, the wrinkles became deeper as development advanced, and frequently the wrinkled ectoderm fell off from the body as small vesicles, although in 0.2 Ca, the spicules eventually developed and short arms grew out.

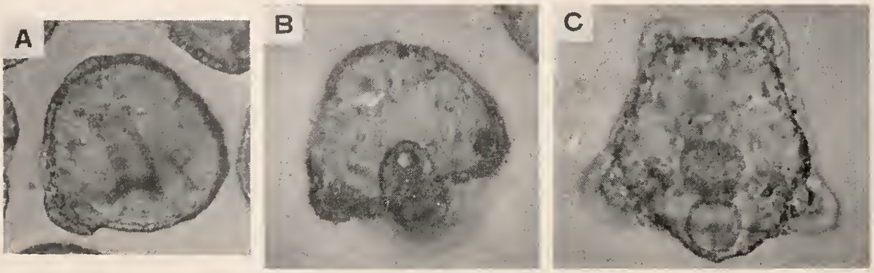

FIGURE 10. *H. pulcherrimus* larvae cultured in Ca-low medium. A, B: 0.1 Ca gastrulae, showing wrinkles of the ectoderm at position of presumptive post-oral arms. A: side view. B: vegetal view. C: 0.2 Ca pluteus, showing post-oral and antero-lateral lobes without rods.

At the stage in which the antero-lateral arms developed in sea water larvae, the ectoderm covering the area of the presumptive antero-lateral arms of 0.1 or 0.2 Ca larvae formed a process which could virtually be considered as an antero-lateral lobe, in spite of the fact that the antero-lateral rod was experimentally suppressed in these larvae (Fig. 10). On transfer back to sea water, this lobe developed into an antero-lateral arm supported by an attenuated skeleton. If several of these slender skeletal extensions grew simultaneously from the central mass, a single branch which elongated in the direction of the lobe acted as a substitute for the antero-lateral rod, but arms never grew out where other branches reached the ectoderm. On continuous culture in 0.1 or 0.2 Ca, the antero-lateral lobe gradually disappeared instead of being cut off from the body. Rulon (1941) reported that *Dendraster excentricus* embryos treated with NaSCN developed into larvae having the general shape of plutei in spite of the lack of the mesenchyme cells and a gut. The Ca-low larva reported here (Fig. 10) is very similar in shape to this larva of Rulon's (Fig 12 in his paper).

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[5] The growth of the skeleton during the third period was frequently better in 0.8 Ca than in sea water.

From the above fact, it can be said that the ectoderm which is to develop into the arm is able to self-differentiate, but for a normal arm to result, a spatial relation between the ectoderm and the skeleton is necessary. This has already been pointed out by Jenkinson (1911), MacBride (1914), Runnström (1931), v. Ubisch (1933) and Hörstadius (1935).

Discussion

As was described in Section II, the higher the concentration of calcium ions, the earlier is the time at which skeleton formation begins. From this fact it is to be expected that the skeletal growth in Ca-high media will be faster than in normal or Ca-low sea water. Such a situation, however, was found to obtain only for a short while after the calcium change (first two hours in Fig. 9). After this period, the skeletal growth was rather accelerated in such low concentrations of calcium as 0.6 or 0.8 Ca, and inhibited by high concentrations. It is still impossible to consider the above facts in terms of the rate of Ca deposition, since it is doubtful whether the apparent size variation of the skeleton is proportional to the quantity of deposited calcium. Abe and co-workers (1954) have reported on calcium deposition by adult oysters which were kept in media of different concentrations of radio-active calcium; if twice as much calcium was provided calcium deposition was slightly increased, while in the medium of half calcium, the deposition of calcium was reduced to nearly one-tenth that found in sea water.

There is another situation to be considered in sea urchin larvae. The spicules of these larvae always grow along a row of primary mesenchyme cells. This fact suggests that the direction of spicule elongation is decided by the mode of arrangement of the mesenchyme cells, and the length of the skeleton is conditioned by the distance to which they extend. The primary mesenchyme cells are further apart in low calcium because of the larger blastocoelic volume, and are brought closer together in high calcium by the smaller size of the blastocoel. On such a basis, it becomes understandable why the apparent growth of the skeleton in 0.6 or 0.8 Ca was rather superior, and that in Ca-high media was inferior to the growth of the controls.

Woodland (1906) and Prenant (1926) suggested from observations of normal skeletal development that a sheath-like structure which is found along the row of primary mesenchyme cells may be a matrix for the skeleton. If this is true, it must be concluded that the apparent length of the skeleton is conditioned by the length of the skeletal matrix which, in turn, is proportional to the distance covered by the longitudinally arranged mesenchyme cells. And further, the tendency that the ratio of length to thickness of the skeleton becomes smaller as the amount of available calcium decreases will find its explanation in the deformation of the matrix resulting from the change in the calcium content of the medium. A detailed description of the skeletal matrix will be reported in the next paper.

The writer wishes to express her sincere thanks to Professor K. Dan and Dr. J. C. Dan for their kind criticism and valuable advice as well as for their assistance in the preparation of the manuscript. The writer's thanks are also due to the director and staff of the Misaki Marine Biological Station for use of the Station's research facilities.

SUMMARY

1. The threshold Ca concentration for the growth of the larval skeleton is lower than that which permits the normal development of sea urchin larvae.

2. The time of the beginning of skeleton formation is retarded by a decrease in Ca concentration from the normal level, while an increased Ca concentration has little effect in this respect.

3. Modification of the Ca concentration of the medium influences the form of the skeleton of sea urchin larvae.

a). In larvae transferred to very dilute calcium before the time of skeleton formation, a mass of unorganized skeletal substance develops.

b). In larvae put into very concentrated calcium from a similarly early stage, several tri-radiate spicules are formed in each larva, but these fail to make a typical skeleton.

c). If the transfer is deferred till the tri-radiate spicules are formed, new skeletal development in the experimental media is continued on the old spicules, and a pluteus skeleton which is similar to the typical one results, though it is much stunted in Ca-low, and slender in Ca-high media.

d). The proportion of the thickness to the length of the skeleton is inversely proportional to the Ca concentration. This is true regardless of the stage of transfer.

e). The elongation of the skeletal rods is inhibited at the extremes of the Ca-series, particularly on the dilute side.

f). The apparent increase in skeletal length is rather better in 0.6 or 0.8 Ca than in high-calcium, except that the contrary situation holds for a short time after transfer.

4. The ectoderm of the presumptive arm may differentiate without any skeleton. However, such differentiated ectoderm finally falls off from the body or disappears unless the supporting skeleton develops.

5. It is suggested that an organic matrix may be formed before deposition of the mineral components of the skeleton; that this matrix is deformed by changes in the calcium concentration; and that such deformation, in turn, influences the rate of growth and the form of the skeleton.

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CYTOKINESIS OF MULTI-SPINDLE CELLS

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Although in cell division it is usually apparent that the position of the constriction furrow is related to that of the spindle, the relationship of the two is not clear. Harvey (1935), working with centrifuged sea urchin eggs, came to the conclusion (p. 296) that, "Since a cleavage plane can come in without relationship to the mitotic figure, it would seem that the mitotic figure itself is an expression of more fundamental physical and chemical changes taking place in the cytoplasm and surface of the egg, and not a cause in itself of the cleavage process." Waddington (1952), working with frog eggs, stated (p. 488) that, "The only evidence, in the present experiment, suggesting an influence by the spindle on the cleavage furrow is that of the few eggs described in which an attempt to move the spindle was followed by an alteration in the course of the furrow. General considerations derived from a study of the cleavage of eggs of many groups certainly suggest that the location of the spindle ultimately determines the position in which the furrow appears." Carlson (1952), on the basis of microdissection studies on grasshopper neuroblasts, held (p. 217) that, "Formation of the cleavage furrow is independent of the spindle and chromosomes, but its position depends to a limited extent on their position at late anaphase." The writer (Roberts, 1955), working with the same material, has presented evidence suggesting the spindle poles, through their relationship to expansions of the polar cell membranes, play an important part in determining the site of the constriction furrow. Cornman and Cornman (1951) have suggested that, upon the dissolution of the nuclear membrane, nuclear material gathers around the centrosomes, is transported by the asters to the cell surface, and is carried by currents to the equator, where it induces the establishment of the furrow. Marsland (1939) and others have described the existence of a "plasma-gel girdle" around the equator of the sea urchin egg prior to cytokinesis without relating it in any way to the spindle.

Cells containing several spindles, usually two to four, are regularly found in preparations of isolated living testicular cells of *Popilius disjunctus*. As is usual in this material, the spindles are invested by long filamentous mitochondria from pole to pole, making the spindles strikingly visible, especially with phase microscopy. Such a multi-spindle condition has been observed in spermatogonia and primary and secondary spermatocytes, which are considerably larger than their single spindle counterparts. Conditions in these cells differ from those seen in dispermic and parthenogenetic marine eggs in that they arise from regularly occurring multinucleate cells, and, ordinarily, each spindle has its own centrosomes and chromosome complement. During cytokinesis each cell divides into two or more cells, de-

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pending on the number of spindles and the orientation of the spindles with respect to each other and to the cell membrane. The patterns of cytokinesis which are observed with varying spindle orientations provide a means for studying the role of the spindle poles in the establishment of constriction furrows.

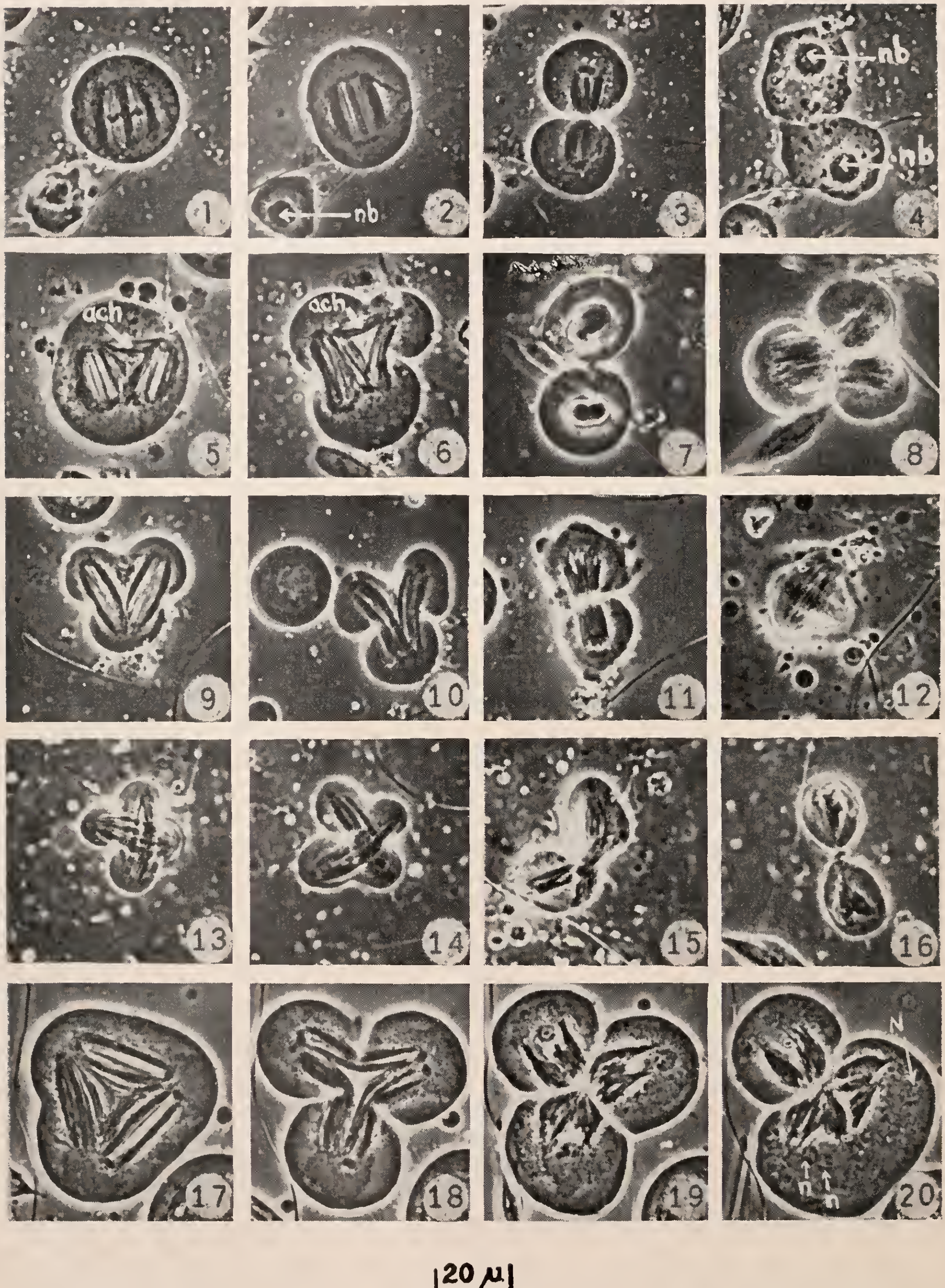
Materials and Methods

Beetles were collected from rotting hardwood logs, brought into the laboratory, and maintained by supplying moist pieces of wood from the logs where they were collected. During the colder months best results were obtained from beetles which had been kept at room temperature, 22° C., for at least one week. Prolonged exposure to temperatures above 30° C. resulted in an absence of dividing cells in the testes. With proper temperature and humidity conditions, dividing cells may be obtained during the entire year.

Several media have been used for the culture of isolated beetle cells. Whole hemolymph is unsatisfactory because it rapidly darkens and becomes cytolytic upon exposure to air. The addition of purified phenyl thiourea prevents blackening and reduces the cytolytic effect, but cell division is adversely affected. A better medium is obtained by flash heating hemolymph, which inactivates those factors responsible for blackening and cytolysis, but also coagulates and precipitates proteins. Cells suspended in such deproteinated hemolymph divide fairly well, but the preparations do not last as long as is desirable. The modified insect Tyrode solution used by Gaulden and Carlson (1951) is satisfactory for preparations which are to be studied for short periods of time, 4 to 6 hours. Best results have been obtained by using a mixture of the insect Tyrode solution with deproteinated hemolymph. The proportions are not critical; usually 50% to 75% deproteinated hemolymph has been used. Preparations made with this medium usually last 18 to 24 hours, and permit observation of two complete cycles of division.

The preparation of this medium presents several difficulties and therefore will be described in some detail. A sterile one-ml. tuberculin syringe equipped with a 1½ inch 26-gauge needle and containing 0.05 ml. of insect Tyrode solution is held in readiness. A beetle, preferably male, is selected and the anterior portion of the head, at the level of the dorsal horn, cut off by one swift cut with heavy scissors. Hemolymph from the severed dorsal vessel accumulates in large clear drops. If the body of the beetle is not squeezed, there is usually no contamination of the hemolymph by material from the digestive tract. If contamination does occur, it can be detected by a brown color in the otherwise colorless fluid, in which case it must be discarded. Occasionally, compression of tissue blocks the dorsal vessel, but this can usually be overcome by slight probing with the syringe needle. The drops of hemolymph are drawn into the syringe and mixed with the Tyrode solution. Ordinarily, enough hemolymph can be obtained to make the total fluid volume in the syringe up to 0.15 ml. It is essential to work rapidly, since the blackening occurs within a few minutes and renders the fluid useless. The fluid in the syringe is discharged into a thin-walled glass bubble for flash heating. The bubbles used are blown from Pyrex tubing and are about 15 mm. in diameter with a capillary neck about 20 mm. in length. The diameter of the neck is just sufficient to admit the 26-gauge needle. After introduction of the fluid, the tip of the neck is sealed

PLATE I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FIGURES 1–20.

in a flame and the bubble is plunged into hot water, just below the boiling point, for 45 seconds. It is convenient to have a beaker of boiling water available and to remove it from the heat while the bubble is being sealed. The fluid within the bubble must be agitated as it enters the hot water and during the entire heating process, otherwise, it will gel and no free fluid can be obtained. In successful preparations the precipitated proteins adhere to the wall of the bubble. After cooling, the bubble should be rotated so that any droplets of distillate are incorporated into the fluid.

Two pairs of testes are present in the adult male beetle. Ordinarily, one pair will supply sufficient material for the amount of medium available. The testes are dissected out and placed in a small dish of Tyrode solution and adherent tissue removed. It is important that the seminal vesicle be removed, otherwise the preparation will be filled with spermatozoa. Nematodes, which are abundant during the summer months, should be removed. The Tyrode solution also has the important function of washing the testes free of unaltered hemolymph which, if carried into the final preparation, has a deleterious effect. The testes are transferred from the Tyrode solution to the center of a sterile microscope slide, carrying over as little fluid as possible. They are then covered with about half the prepared medium, which has been withdrawn from the bubble with a sterile one-ml. tuberculin syringe and a 26-gauge needle. The testes are squashed against the slide, using flat-tip cover-slip forceps. Thorough squashing is necessary to rupture the cysts of the testis, but a scouring motion, which destroys many cells by its shearing action, should be avoided. The remainder of the medium is discharged over the squashed testes and the slide tilted so that the fluid and cells drain away from the squashed tissue. This cell suspension is then drawn into the same syringe, leaving the fibrous debris on the slide.

One-centimeter squares are outlined on coverslips with vaseline extruded from a 26-gauge needle. Each square is flooded with the prepared cellular suspension

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FIGURES 1–4. Division of a secondary spermatocyte containing two parallel spindles. A normal spermatid containing a single nebenkern (nb) is shown at the lower left of Figure 2. The two large spermatids resulting from the division are shown in Figure 4.

FIGURES 5–7. Division of a secondary spermatocyte containing two divergent spindles. A well developed achromosomal spindle (ach) connected the divergent poles. A second furrow formed between the divergent poles but soon regressed. Two large spermatids resulting from the division are shown in Figure 7.

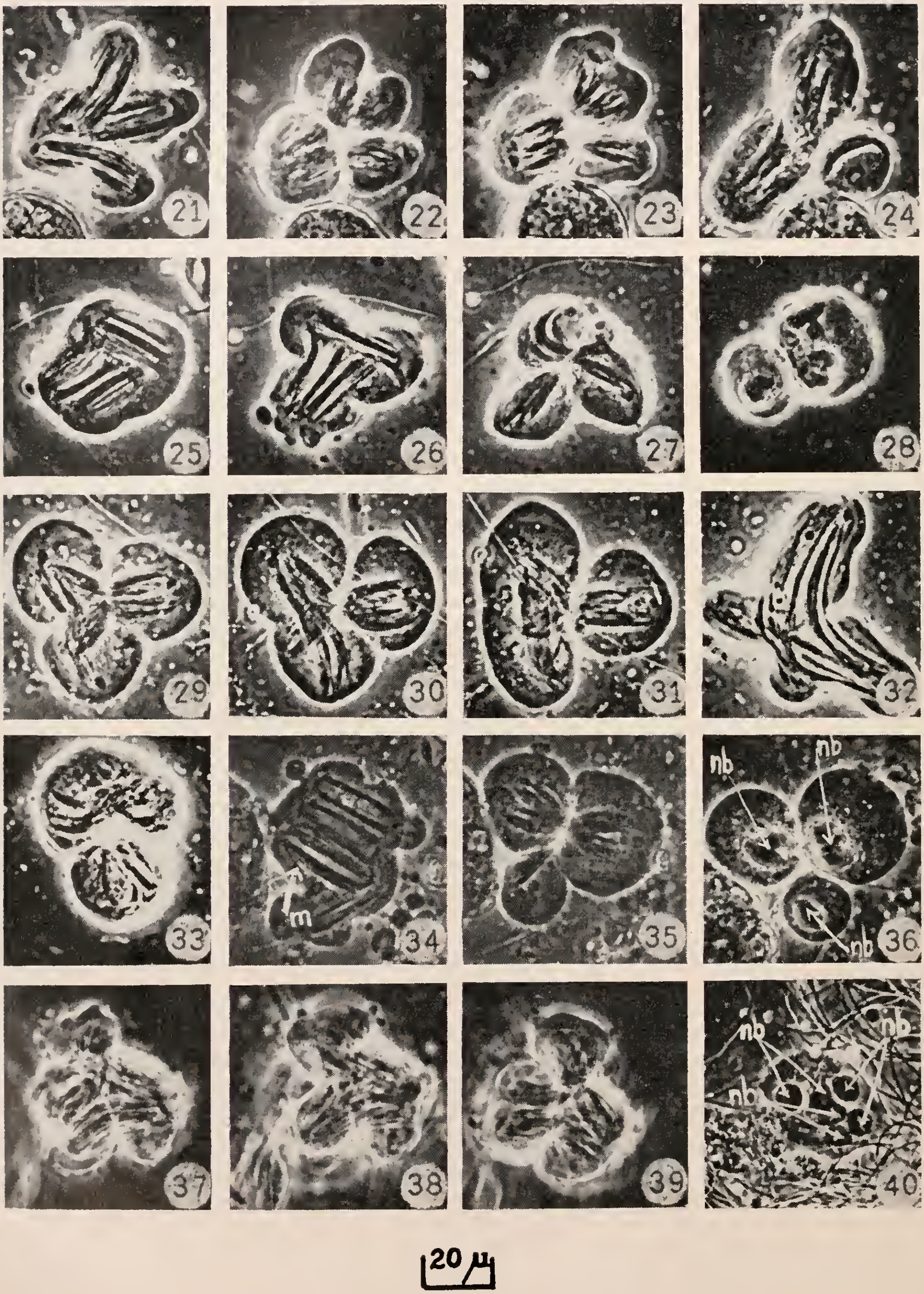
FIGURE 8. Three daughter cells resulting from division of a primary spermatocyte containing two divergent spindles.

FIGURES 9–11. Division of a primary spermatocyte containing two divergent spindles. There was no evidence of cross-connections between the divergent poles. A furrow developed between the divergent poles and advanced halfway before regressing.

FIGURES 12–16. Division of a primary spermatocyte containing two spindles at right angles to each other, in different planes and not intersecting. There was no evidence of cross-connections between the poles. Furrows developed between the poles and the cell appeared to be dividing into four. As the furrows impinged on the spindles their orientation changed so that they came to be approximately parallel and the cell divided into two.

FIGURES 17–20. Cytokinesis of a primary spermatocyte (?) containing three spindles. Furrows developed between the poles, in the equatorial region. In late cytokinesis one furrow regressed, and two of the daughter cells fused. In Figure 20 it can be seen that in one case the two chromosome groups fused, forming a large nucleus (N) and in another the groups remained separate, forming two small nuclei (n).

PLATE II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FIGURES 21–40.

and the excess fluid withdrawn into the syringe. This gives a relatively flat film whose thickness can be adjusted as required. The coverslips are inverted over depression slides and sealed with vaseline or heavy mineral oil. Two types of depression slides have proved especially useful. One (Fisher-Littman) is of the same thickness as a standard microscope slide and has a shallow circular depression with a flat polished bottom. This slide permits the use of oil immersion phase objectives. A specially made plastic well slide has been most commonly used. This slide is too thick to permit the use of oil immersion objectives, but has the advantage that it takes 22 × 50 mm. cover-slips on which three vaseline squares can be outlined. The relatively large air space is compensated for by recesses on all four sides into which cotton, wet with Tyrode solution, can be packed.

Divisions were studied with dark contrast phase optics (Spencer) using long focal length phase plates, to permit study of thick preparations. In the writer's experience with living cells, the long focal length phase plates have proved to be clearly superior, regardless of the thickness of the preparation. Light intensity was controlled by a variable transformer and overheating of the slides was avoided by using a copper sulfate water cell and three heat-absorbing filters (Corning). Observations and records were made at room temperature, about 22° C.

Observations

The observations reported are based on spindle orientation in living cells as revealed by oriented filamentous mitochondria. The spindle fibers are not visible. A comparison with stained sections indicates that the structure observed in living cells is a fairly accurate representation of the actual distribution of fibers. The chromosome-bearing spindles are faithfully visualized, but cross-connections (achromosomal spindles) between the poles of adjacent chromosome-bearing spindles are less reliably indicated. The filamentous mitochondria, which are preserved and stained by those methods used to demonstrate fibers, tend to obscure the fibers along which they are oriented. Wherever oriented mitochondria indicate the existence of such cross-connections, fiber connections actually exist, and the relative abundance of fibers is correlated with the abundance of oriented mitochondria. In the absence of oriented mitochondria, a few weak fibers may be present or there may be none.

Figures 21–24. Cytokinesis of primary spermatocyte containing three divergent spindles. There was no evidence of cross-connections between the divergent poles. Furrows developed between the divergent poles, and the cell appeared to be dividing into four. Strong blebbing, Figures 22, 23, was followed by regression of one furrow, and the cell eventually divided into three.

Figures 25–28. Cytokinesis of a secondary spermatocyte containing three spindles. After cytokinesis was virtually complete, Figure 27, two daughter cells fused, giving rise to one large and one small spermatid.

Figures 29–33. Cytokinesis of primary spermatocytes(?) with three spindles.

Figures 34–36. Cytokinesis of a secondary spermatocyte containing four spindles, three parallel and the fourth divergent. Although a mitochondrion (m) was present in the area between the divergent poles, no achromosomal spindle was visible. Two large and one small spermatid resulted. Note the relative size of the nebenkerne (nb).

Figures 37–39. Cytokinesis of a cell containing six spindles. Three daughter cells resulted.

Figure 40. Spermatid containing six nebenkerne (nb). This cell was observed immediately after the preparation was made, far too short a period for this to be the result of the procedure used.

Figures 1–16 illustrate cytokinesis of cells containing two chromosome-bearing spindles. In the simplest situation the two spindles, each with its own chromosome complement, were parallel and close together. The furrow formed in the equatorial position and the cell constricted into two daughter cells as though only one spindle were present (Figs. 1–4). In some cases the two chromosome groups in each polar region fused to form one nucleus. In others, two nuclei were formed. In many double spindle cells the spindles were in the same plane but lay at an angle to each other. Under these conditions a furrow developed in the equatorial region, and a second furrow invariably developed between the divergent poles. The formation of the second furrow did not depend upon the presence of cross-connections between the divergent poles, as far as could be judged from the presence of oriented mitochondria. In some cases a well developed achromosomal spindle was present (Figs. 5–6), and in others (Figs. 9–10) there was little or no evidence of cross-connections. The fate of the second furrow depended primarily upon the distance between the divergent poles and not on the presence of fiber connections between these poles. Where the poles were only slightly separated, the second furrow soon regressed and the cell constricted into two daughter cells (Figs. 4, 7). Regression often occurred after cytokinesis was virtually complete, resulting in the fusion of two or even all three of the daughter cells. A few double-spindle cells have been observed in which the two spindles lay at right angles to each other, in different planes, and not intersecting (Figs. 12–16). During cytokinesis furrows developed between each of the four poles so that the cell appeared to be dividing into four daughter cells. In the cell illustrated there was no evidence of cross-connections between the poles. In cells of this type, as the furrows impinged upon the spindles their orientation changed so that they came to be approximately parallel, and the cell eventually divided into two.

Figures 17–33 illustrate cytokinesis of cells containing three chromosome-bearing spindles. Various spindle orientations occurred. In some, the three spindles lay in the same plane and formed the sides of an equilateral triangle (Fig. 17). Furrows developed in the equatorial region of each spindle and developed so that division into three daughter cells was virtually completed (Fig. 19). In late telophase, after re-formation of interphase nuclei, two of the daughter cells fused into one large cell (Fig. 20). In another case, first observed in early cytokinesis, the three spindles were arranged as shown in Figure 21. There was no evidence of cross-connections between adjacent poles. Furrows developed between each of the diverging poles and across the equatorial region of all three spindles. The cell appeared to be dividing into one large and three smaller daughter cells. Later, strong blebs developed on two adjacent incipient daughter cells (Figs. 22, 23), the furrow between them receded, and the cell finally divided into two large and one small daughter cell (Fig. 24). It is noteworthy that the appearance of strong blebs adjacent to a furrow always resulted in the regression of that furrow. In another case two of the three spindles were parallel and the third at approximately right angles to the other two (Fig. 25). Furrows formed in the equatorial regions and advanced until, in late cytokinesis, two incipient daughter cells fused so that one large and one small daughter cell were produced (Fig. 28). Other spindle orientations in which the spindles were, in part, in different planes are illustrated in Figures 29–31 and 32–33. As in the other cases described, furrows developed between the divergent poles without regard to the presence or absence of cross-connecting fibers.

Cells containing from four to six chromosome-bearing spindles have been observed. Cytokinesis in such cells is complex and difficult to follow (Figs. 37–39). A simpler case of a cell with four chromosome-bearing spindles is illustrated in Figures 34–36. It is apparent here also that furrows develop between spindle poles and not necessarily in relationship to the equatorial region of the cell or spindle.

Discussion

It is evident in this material that the site of furrow formation is related to, and apparently dependent upon, the position of the spindles. It has been repeatedly observed that furrows form approximately halfway between spindle poles and not necessarily in relation to the equators of the spindles. The presence of spindle fiber tracts (chromosomal or achromosomal spindles) between the poles does not appear to be essential for the formation and further development of a furrow. Since in the usual situation the equator of a spindle does lie halfway between spindle poles, the commonly observed relationship between furrow and spindle equator can be explained on this basis.

Bajer (1954) has described a quite similar situation in primary spermatocytes of the snail *Cepea hortensis*. Although his figures of trinuclear and tetranuclear cells show only the chromosome groups and not the spindles, it is clear here also that furrows form between spindle poles and not in relationship to the equators. His description of cytokinesis of binuclear cells without division of the nucleus is especially striking. In these cells each nucleus reached metaphase, with the two spindles parallel and widely separated. After a "long" metaphase with "quiverings" of the plate, the furrow formed and advanced between the two spindles without cutting through either. The cell divided into two and interphase nuclei were reconstituted from each metaphase configuration.

These observations support the idea that the spindle poles initiate furrow formation, possibly through a "furrow determiner" as postulated by Cornman and Cornman (1951), or a "structural agent" as proposed by Swann (1952) and Mitchison (1952) and modified by Roberts (1955). They do not appear to provide an adequate basis for deciding between these alternatives. Bajer's observations on cytokinesis of binuclear cells without nuclear division would appear to support the view of the Cornmans that nuclear material released upon the dissolution of the nuclear membrane may act as a furrow determiner. It has been noted that in multi-spindle cells the distance apart of divergent poles influences the furrow which develops between them. When the distance is sufficiently great, the furrow advances and may succeed in dividing the cell. This does not appear to support the view of the Cornmans, since it would seem that the accumulation of large quantities of "furrow determiner" in the small area between the poles should result in the development of a strong furrow. Swann and Mitchison postulate that the furrow is established by an initial contraction of the cell membrane between the "structural agent" induced polar membrane expansions, and that spread of the expansions into the furrow actively advances it. In this material it has been observed that when large blebs, which are membrane expansions of a sort, occur in the furrow area, the furrows usually regress. Further, it would seem that proximity of a developing furrow to the point of release of the expansion inducing substance should facilitate the further growth of the furrow. There is no difficulty if the furrow, after a critical stage in its establishment, is autonomous and continues to develop by contraction or autocatalytic growth as suggested by the Cornmans. This is supported by the work of Waddington (1952), Harvey (1935), Carlson (1952) and others. The observation that the furrow developing between spindle poles separated by only a short distance soon regresses could be explained as the result of the initial contraction being obliterated by the approaching waves of expansion before the critical stage is reached.

In the present work, as in that of the Cornmans, there is evidence of a critical stage just at the completion of cytokinesis. Where multiple furrows are present, it is usual that one or more furrows regress, and it is rarely that these cells succeed in dividing into more than two daughter cells. This was also true of the multinuclear cells described by Bajer. Certainly adequate spindle material is available, and it is not likely that this is a factor as suggested by the Cornmans. Bajer's observations indicate that spindle material is not required for the completion of cytokinesis, since constriction was completed without the furrow cutting through a spindle.

It is evident that some unusual chromosomal relationships must result from divisions such as those described. These have not thus far been analyzed. Preliminary attempts to do chromosome counts by the squash method have not been satisfactory. The chromosomes are so small that the pressure required to spread them sufficiently for counting made cell boundaries unrecognizable, and it was not possible to determine whether adjacent chromosome sets came from the same or different cells.

The multi-spindle cells studied do not result from the procedures used. Prometaphase lasts approximately three hours, and anaphases and telophases have been observed within a few minutes after making a preparation. Living spermatids have frequently been observed to receive as many as two sets of chromosomes (Figs. 4, 7, 28) and occasionally as many as three (Fig. 36). In the normal spermatid the single nebenkern divides into two before the mulberry stage is reached, so that not more than two nebenkerne are normally seen. Spermatids containing at least six nebenkerne (Fig. 40) have been observed in freshly made preparations.

SUMMARY

1. Patterns of cytokinesis have been studied in living multi-spindle cells obtained from beetle testes.

2. Furrows develop between spindle poles, whether or not connected by spindle fibers, and not necessarily in relationship to the equator of a spindle.

3. The further development of furrows is related to the distance apart of spindle poles. If the spindle poles are close together, the furrow soon regresses; if the poles are sufficiently far apart, the furrow advances and may succeed in dividing the cell.

4. When large blebs develop in the furrow area, the furrow regresses.

5. The observations reported are discussed in the light of current theories of cytokinesis.

6. Details of the unusual chromosome distributions which result have not been analyzed in detail.

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RE-EXAMINATION OF THE PRODUCTION OF CYCLOPIA IN FUNDULUS HETEROCLITUS WITH MAGNESIUM CHLORIDE AND ETHYL ALCOHOL[1]

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During three seasons (1948, 1949, 1955) attempts were made to produce perfect cyclopean fish for anatomical and physiological studies. Because of commonly accepted, misleading statements in the literature, much time was wasted at first. In brief, the large percentages of cyclopean embryos reported after $MgCl_2$ treatment by Stockard (1907, 1909) are not readily obtainable by this method; but instead, very large numbers of experimental animals must be handled to secure even a few hatched, perfect cyclopean fish.

The first purpose of this paper is to present quantitative data on this negative aspect of the problem. Such data have been lacking in previous reports, although McClendon (1912) has pointed out that the high percentages obtained by Stockard were not obtained by him. A second, positive purpose is to suggest that specific temperature in combination with the chemical treatment is of prime importance. Third, since ethyl alcohol treatment gives a large percentage of cyclopean embryos (although they are extremely unlikely to hatch), precise data on the effective concentrations are presented.

MAGNESIUM CHLORIDE

In work with this salt, gram molecular stock solutions of $MgCl_2 \cdot 6\ H_2O$ were made at the start of each experiment. In the manner of Stockard, 18 parts of this solution were added to 42 parts of sea water, 19 parts to 41 parts of sea water, etc. These were designated by Stockard as 18/60 *M*, 19/60 *M*, etc. The fact that they do not truly bear this relation to a molar solution is not especially significant. If a series of concentrations from completely ineffective to completely lethal is used, the optimum concentration will be included. The method is convenient and allows close comparison with Stockard's work.

In 1948 the experiments used fish obtained May 21st from a live-bait dealer in Boston, and sea water was obtained off the coast away from the city and stored in glass carboys. A liter of stock solution was made with 203.33 grams of $MgCl_2 \cdot 6\ H_2O$ in distilled water. About half an inch of properly diluted solution was added to each batch of eggs in 4″ finger bowls from 3 to 18 minutes after insemination. The bowls were stacked to prevent undue evaporation and stored in

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TABLE I

1948 $MgCl_2$ experiments

Solution	Dead or undeveloped	Two-eyed embryos	Cyclopean embryos	Double embryos
18/60 $MgCl_2$ (2 bowls)	675	7	0	0
19/60 $MgCl_2$ (8 bowls)	1081	261	2	1
20/60 $MgCl_2$ (2 bowls)	338	31	0	0
Control (sea water)	62	0	0	0

an aquarium room with a temperature range of 16 to 21° C. Solutions were replaced every 48 hours. Undeveloped but dead eggs were counted and removed after the first two days. After 12 days the embryos were preserved and inspected under the compound microscope. The results are presented in Table I.

In 1949 eggs were fertilized at Woods Hole from June 16th to July 5th. The season was early and it became increasingly difficult to find fish which had not already shed. At first fish came from the waters around Woods Hole, but during the last week some were obtained from Barnstable on the north side of Cape Cod.

Sea water from the outlets on the water tables in the laboratory was added to a depth of half an inch, five to ten minutes after fertilization, and replaced by magnesium chloride solutions when the embryos were in the four- to eight-cell stage. Bowls were tipped up and drained as dry as possible in changing solutions, to avoid

TABLE II

1949 $MgCl_2$ experiments

Solution	Died during development	Normal two-eyed embryos	Cyclopean embryos
A. Stock solution made up in sea water			
15/60 $MgCl_2$	3 (12%)	23	0
18/60 $MgCl_2$	21 (36%)	37	0
22/60 $MgCl_2$	35 (81%)	8	0
B. Stock solution made up in distilled water			
15/60 $MgCl_2$	7 (18%)	33	0
16/60 $MgCl_2$	8 (18%)	37	0
18/60 $MgCl_2$	4 (7%)	51	0
19/60 $MgCl_2$	30 (26%)	84	0
20/60 $MgCl_2$	42 (41%)	60	0
21/60 $MgCl_2$	70 (77%)	21	0
23/60 $MgCl_2$	76 (77%)	23	0
25/60 $MgCl_2$	51 (100%)	0	0
C. Sea water controls (adults for second control came from closed brackish pond)			
	2 (6%)	30	0
	52 (48%)	56	0

further dilution. The solutions were replaced every 12 hours; embryos were kept in the solutions for 48 to 72 hours, until the condition of their eyes was clear from inspection under the compound microscope. In contrast to the previous experiments, unfertilized eggs were removed within several hours of insemination; therefore only eggs which passed through the initial cleavage stages were tabulated. Finger bowls were stacked on a desk, and water temperature in the bowls was recorded at times scattered through the day and night. Of 50 recorded temperatures, 28 fell between 23° and 25° C. Only two were below 22° C. (20.5° and 21.0°) and four above 26° C. (26.25°, 26.5°, 27.25°, and 27.5°).

The effect of magnesium chloride dissolved in sea water alone (Stockard, 1907) was tested; 203.33 grams of $MgCl_2 \cdot 6\ H_2O$ were dissolved in one liter of sea water.

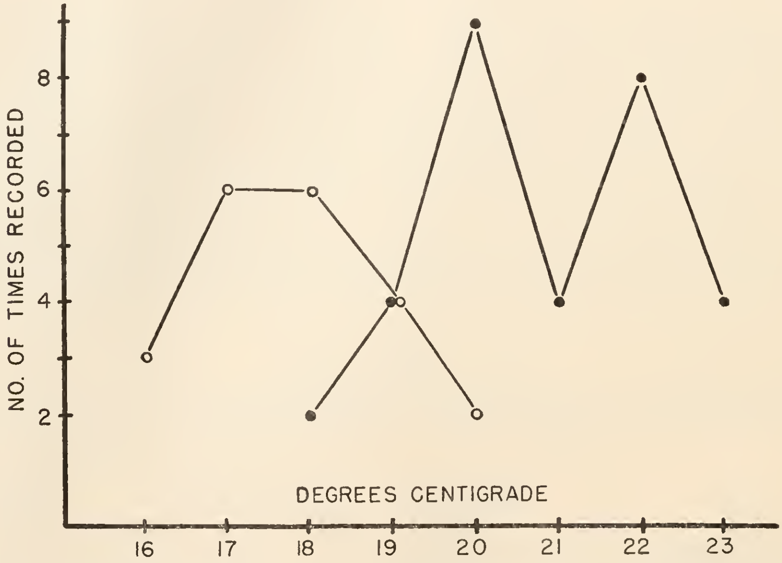

FIGURE 1. Recorded experimental dish temperatures, 1955. Temperature was recorded usually four times a day, and diurnal fluctuation in the laboratory room was kept as low as possible. Open circles represent recordings before June 12th, solid circles represent recordings after June 12th.

This is not a molar solution but the salts in sea water would cause inexactness anyway. Closer measurement seemed unnecessary since a range of concentrations was to be employed. This stock solution was then added to sea water in the same manner as the distilled water solutions of magnesium chloride had been. In another series, a gram molecular solution of $MgCl_2 \cdot 6\ H_2O$ in distilled water was titrated colorimetrically. This solution was added to sea water and the eggs and bowls were handled as described. The results of these two series are shown in Table II.

In 1949 because of the failure of the magnesium chloride treatment to produce any cyclopean embryos, more extensive series were not run. Alcohol treatment instead was used to secure cyclopeans for anatomical study (Rogers, 1952). The alcohol method had the disadvantage of not allowing embryos to develop well enough to hatch when used in concentrations that would produce cyclopia. Accordingly, when free-swimming cyclopean fish were desired for physiological tests in 1955, very large series of $MgCl_2$ experiments were carried out. From the results of 1948 and 1949 there was a suggestion that temperature might have an effect. The hurricane-flood damage of the previous season at Woods Hole prevented use of the cold rooms for some time in 1955, so experiments were carried out on a laboratory table at first. Room temperature was kept as constant as diurnal fluctuation allowed and experimental dish temperature was recorded at times scattered through the day and night. These experiments fall into two groups: those in which the embryos passed the critical eye-determining period during cold weather between June 5th and 11th (series A), and those after a storm temporarily prevented obtaining adults, or the warmer period between June 12th and 19th (series B). The recorded temperatures during these two periods are plotted in Figure 1.

TABLE III

1955 $MgCl_2$ experiments

No. of bowls	Solution	Died during development	Normal two-eyed embryos	Cyclopean embryos	Other abnormals	% of living that were abnormal
			Series A			
3	15/60 $MgCl_2$	54—(31%)	120	0	0	
19	18/60 $MgCl_2$	682—(28%)	1713	38	22	3.4%
23	19/60 $MgCl_2$	864—(28%)	2139	58	60	5.2%
11	20/60 $MgCl_2$	1052—(51%)	967	17	38	5.4%
2	21/60 $MgCl_2$	136—(52%)	124	2(?)	1	
3	24/60 $MgCl_2$	514—(95%)	26	1(?)	1	
1	25/60 $MgCl_2$	153—(88%)	20	0	0	
2	Sea water controls	46—(10%)	395	0	0	
			Series B			
27	19/60 $MgCl_2$	Not recorded	3488	79	15	2.6%
			Series C			
	19/60 $MgCl_2$					
24	10° C.	1429—(100%)	0	0	0	
24	18° C.	292—(20%)	1070	42	26	6.0%
24	25° C.	137—(11%)	1156	3	7	0.9%

A third series was run in temperature-controlled chambers from June 22nd to July 3rd (series C). The 10° cold room varied from 6° to 10° C., but was between 9.5° and 10° C. most of the time. The 18° room varied between 16.5° and 19.1° C.; the 25° chamber, between 25° and 26° C. The two colder chambers were sizeable rooms, whereas the 25° chamber was the size of a household refrigerator. Twenty-four bowls were kept at each temperature. In 16 cases batches of eggs, each from single pair matings, were divided and a third of each batch placed in a bowl at each temperature, to have a control on hereditary disposition as far as possible. The remaining eight bowls at each temperature had to be made up by dividing into thirds, batches of eggs combined from stripping many adults. After the eyes were determined, embryos were returned to sea water and kept at laboratory room temperatures (around 24° C. at that time).

Methods of handling all the bowls followed those described for 1949. A gram molecular solution of $MgCl_2 \cdot 6\ H_2O$ in distilled water was used in every case as the stock solution; no sea water stock solutions of $MgCl_2$ were employed. All of the 16,987 eggs were inspected under the dissecting microscope at least twice, once in the cleavage stages, when additional, non-developing eggs were removed, and again to classify the embryos after the eyes had developed. In addition, all 410 abnormal embryos were kept in sea water for a further period so that they could be more certainly classified after further development, although even then reasonable doubt sometimes remained. Complete records were kept on every bowl; the salient features of the 64 pages of data are summarized in Table III.

Alcohol

Stockard (1910) prepared mixtures on a percentage basis and found that from 3 to 9% ethyl alcohol in sea water produced viable cyclopean embryos, although he stated (p. 369) that "only a few of the alcoholic specimens ever develop sufficiently to hatch and swim about as do the Mg embryos." In his experiments batches of 60 to 100 eggs were exposed to 60 cc. of solution "for (not) more than twenty-four to thirty-six hours" following the eight-cell stage and then returned to sea water. Mention was not made of the methods employed, if any, to prevent evaporation.

In the present work tests were made to determine what precautions were necessary for the prevention of rapid evaporation. At 20° C. the partial pressure of ethyl alcohol in a 10% solution in water (by weight) is 28% of the total vapor pressure of the solution and at 40° C. is 34% (International Critical Tables). Initially comprising only 10% of the solution, the alcohol will contribute almost a third of the initial evaporation. The percentage of alcohol in the solution will fall rapidly if evaporation is allowed to take place. The salts in sea water will have no significant effect on the situation (personal communication from Professor G. B. Kistiakowsky). Stacking of finger bowls seemed inadequate to prevent evaporation of this magnitude and sealing the bowls would be time-consuming; a glass plate larger than the finger bowl was therefore laid over the aperture of each bowl. As a test of the effectiveness, covered and uncovered bowls were left 17 hours at 20.5° C. in a laboratory room. Each initially contained 55 cc. of a 10% (by volume) solution of absolute ethyl alcohol in sea water. At the end of the period the covered bowl contained 54.75 cc. (the difference from the initial volume being within the limits of error of measurement); the uncovered bowl contained only 48 cc. Since in the experiments solutions were replaced every 12 to 17 hours, and the dishes were covered, evaporation was not significant.

The experiments with alcohol were carried out in much the same way as those with $MgCl_2$. Unripe and unfertilized eggs were removed at the start of the experiment so that only eggs that were initially developing were tabulated. The embryos were returned to sea water in the 1949 series after 24½ to 33½ hours, and in the 1955 series after 51 to 53½ hours of exposure to the alcohol mixtures. In the latter case the embryos were left in the alcohol solutions until they could be seen to be developing eyes in the less lethal concentrations. Experiments in 1949 were at temperatures most frequently between 23° and 25° C. as noted for the $MgCl_2$ experiments, and in 1955 entirely between 21.5° and 22.8° C. Lethality of the solutions is summarized in Table IV.

It should be noted that the two embryos counted as having survived the 5% alcohol treatment in reality failed to axiate. In these two cases the yolk spheres did not disintegrate and unorganized masses of cells with a few chromatophores developed upon their surfaces. The 5% alcohol mixture thus for all practical purposes was lethal. This held true when it was used for 32 hours or for 51½ hours (the two surviving eggs were in the latter group).

The 2% and 2½% solutions (1,268 embryos) produced only about a dozen perfect cyclopeans, which did not hatch. A dozen embryos in these bowls did hatch, including one synophthalmic embryo. The 3% solution proved best for production of cyclopia while still allowing moderately good development of body form. In the 1949 series with 3% alcohol, embryos were tabulated individually, the living embryos giving the following totals: 128 cyclopeans including some with a single optic cup but two lenses, 43 monophthalmics, 60 two-eyed embryos including some synophthalmics which had two optic cups, each with lens and pupil, and 26 individuals that could not be classified. None of the embryos treated with 3% alcohol hatched. The 4% solution yielded an even higher percentage of cyclopean embryos among the survivors, but general body development was so inhibited and so many of the eyes were greatly reduced in size that results did not compare favorably with those at 3% concentration. The 4½% solution allowed only abortive attempts at axiation, and as noted above, the 5% solution was lethal.

TABLE IV

1949 and 1955 alcohol experiments

No. of bowls	% of alcohol	Total embryos	% embryos dead before eyes developed
4	2%	650	5%
3	2½	618	4
9	3	692	29
4	4	697	73
3	4½	608	89
4	5	869	99.8
		4,134	

DISCUSSION

The first point that is demonstrated by the present data is that the $MgCl_2$ method will not ordinarily give large percentages of cyclopean embryos. McClendon (1912) stated (p. 139), "In numerous experiments covering an entire season, I failed to obtain as high a per cent of cyclopia with magnesium chloride as recorded by Stockard." Stockard (1909) admitted difficulties in repeating his initial experiment. Yet McClendon's work has largely been overlooked because of its negative character. Stockard classified all grades of synophthalmia as cyclopia, and yet the percentages of *all* abnormals in the best instances in the present experiments remain at about one-tenth of the fifty per cent cyclopia reported by Stockard. In Table III the numbers of cyclopean embryos probably are too high, since whenever there was doubt in cases where the eye seemed to be cyclopean but was so reduced as to make it difficult to be certain, the embryo was classified "cyclopean" rather than "other abnormal." The totals for *all* abnormals, however, are accurate.

The chances of obtaining free swimming cyclopean fish are not as good as the tabulated figures would make them appear to be. Only three cyclopean and four synophthalmic embryos hatched in the 1955 experiments. In addition, 21 monophthalmics, 17 with one eye partially reduced, and three anophthalmics hatched. All of these came from Series A except for three monophthalmics and two with one eye partially reduced from Series B, and one synophthalmic and two monophthalmics from untabulated bowls started after the temperature-controlled experiments and upon which individual records were not kept. No abnormals from the temperature-controlled experiments (Series C) hatched, a fact for which no explanation is apparent.

The influence of temperature in the $MgCl_2$ work now seems clear. Loeb (1915) and Kellicott (1916) reported some normally developing Fundulus embryos after eggs had been exposed in the early stages for a time to 7° C., and 8 to 10° C., respectively. Many embryos died or were markedly abnormal after such exposure, however. In the present work it is clear that the 19/60 $MgCl_2$ mixture couples its effect with that of cold in an additive way, for not one embryo survived of 1,429 exposed to 10° C. in the $MgCl_2$ solution (Series C, Table III). Most of them reached the late blastula stage before disintegrating. At temperatures for the most part of 17 to 18° C., between 5 and 6% of 3,395 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series A and C). At temperatures for the most part of 20 to 22° C., 2.6% of 3,582 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series B). Finally at temperatures between 25 and 26° C. only 0.9% of 1,166 surviving embryos in 19/60 $MgCl_2$ were abnormal (Series C). Unfortunately there were no means to test temperatures between 11 and 16° C. There remains the possibility that the optimum temperature for obtaining cyclopean embryos in the $MgCl_2$ solutions lies within this range.

In addition to the temperature differences there is the possibility that differences in hereditary susceptibility may cause variation in results. The incidence of any abnormality was so low in the 16 bowls kept at 25° C., for which there were control bowls kept at 18° C., that no significant results were obtained. Nevertheless, one cannot fail to be impressed with the great variation that does occur among batches of eggs handled in exactly the same way under the same conditions. The 19/60 $MgCl_2$ tests in Series A, Table III, are a good example. Of 58 cyclopean embryos, 46 occurred in three bowls, and of the total of 23 bowls, 14 contained no cyclopeans at all.

The results of the alcohol work indicate that Stockard did not control evaporation to any extent since he found 3% to 9% alcohol to be effective in producing living cyclopean embryos, and the present work shows 5% alcohol to be lethal if that concentration is maintained. Desired results may be obtained in percentages of alcohol steadily decreasing with time, but conditions cannot be repeated without control of humidity, temperature, and air currents.

SUMMARY

1. Under the conditions of the present $MgCl_2$ experiments, which followed those of Stockard as closely as possible except for temperature control, 2.0% of 11,912 surviving embryos were cyclopean; 0.3% were perfect cyclopean with an eye close to normal size, of which only three individuals hatched.

2. The optimum concentrations of $MgCl_2$ at the temperatures of these experiments were those which Stockard designated 18/60, 19/60, and 20/60 *M*.

3. With 19/60 *M* $MgCl_2$ solution, 10° C. proved lethal, while 17 to 18° C. was the optimum temperature used, with effectiveness falling as the temperature increased to 26° C. It was not possible to test temperatures of 11 to 16° C.

4. At temperatures of 21.5 to 25° C., 3% alcohol in sea water was the most effective in producing cyclopean embryos with moderately well developed body form; 4½% alcohol allowed only abortive axiation and 5% alcohol was lethal.

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MATING TYPES IN OXYTRICHA AND THE SIGNIFICANCE OF MATING TYPE SYSTEMS IN CILIATES[1]

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Sonneborn's (1937) discovery of mating types in *Paramecium aurelia* made possible a new approach to the classical and perplexing problems of sexuality, inheritance, and life cycles in ciliated protozoans. Mating types were later found in other species of this genus (Sonneborn, 1938; Jennings, 1938; Giese, 1939; Gilman, 1939) and in *Euplotes* (Kimball, 1939), *Stylonychia* (Downs, 1952), and *Tetrahymena* (Elliott and Gruchy, 1952; Nanney and Caughey, 1953). The present communication reports mating types in a fifth genus, *Oxytricha*. The mating behavior and some aspects of the life cycle of *O. bifaria* will be described. Finally, speculations as to the functions and significance of the known systems of binary and multiple mating types in ciliates will be presented.

MATERIALS AND METHODS

Specimens of fresh water collected from natural sources were found to contain the hypotrich, *Oxytricha bifaria*. Four stock cultures, each representing the progeny of a single cell, formed the initial material for this investigation. A list of the stocks and their sources follows: stock 4 from Bloomington, Ind.; stock 14 from Ann Arbor, Mich.; stocks 24A and 24C from Locksley Station, Pa. These cultures can be induced to conjugate with one another and, together with their sexual progeny, furnish nine mating types to be described below. Additional collections provided new stocks belonging to one or another of these mating types.

In general, the methods described for handling and culturing *Paramecium aurelia* (Sonneborn, 1950) were successfully adopted for studies of *Oxytricha*. Mass cultures and isolation lines were routinely maintained in lettuce or Cerophyl infusions inoculated with *Aerobacter cloacae*. Under these conditions the present strains of *Oxytricha* have given, after three years of observation, but few indications of cyclic periods of depression or of senescence and death reported for other pedigrees (see Jennings, 1929). But it is important to note that some stocks and hybrid clones (obtained from crosses between the original stocks) lost their initial vigor, became senile and finally died. To induce conjugation, diverse cultures were provided sufficient nutrient to allow 0.5 to 1.0 fissions per day, and were mixed in a depression slide when they had exhausted most of the available bacteria. Mating began about two hours after the mixture was made; if well-fed cultures were mixed, the occurrence of conjugation was delayed until the food supply had been further exhausted. When one or both of the cultures were allowed longer periods of

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starvation before mixture, the cells usually became cannibalistic. Cannibals soon attained abnormally large sizes and could not be induced to conjugate. Under optimal conditions, 25 to 95 per cent of the cells formed pairs. Conjugation can apparently take place at any hour of the day and at temperatures ranging from 17 to 32 degrees C.

Nuclear reorganization in the present strains is apparently limited to conjugation; attempts to induce autogamy by limiting the food supply of mature clones proved unsuccessful.

Results and Discussion

In *Paramecium,* true conjugation can take place only between cells of complementary mating types. But in *Euplotes,* cells of a given mating type will conjugate when exposed to filtrates from cells of other mating types (Kimball, 1939) and, for this reason, mixtures of diverse cultures may come to contain induced "selfing" pairs together with other pairs consisting of cells of complementary mating types.

Table I

The breeding system in O. bifaria

(*The plus signs signify that conjugation normally occurs in mixtures of the types indicated*)

	I	II	III	IV	V	VI	VII	VIII	IX
I	−	+	+	+	+	+	+	+	+
II		−	+	+	+	+	+	+	+
III			−*	+	+	+	+	+	+
IV				−	+	+	+	+	+
V					−	+	+	+	+
VI						−	+	+	+
VII							−	+	+
VIII								−	+
IX									−

* Small numbers of selfing pairs frequently observed.

Pairs formed following the mixture of diverse cultures of *Oxytricha* regularly consist of cells of diverse types. This conclusion is based on the following facts: (1) Animals cultured in lettuce infusions for a few days appear darker than others grown in Cerophyl infusions. Appropriate mixtures of "dark" and "light" cells provided conjugations in which each pair consisted of a dark cell mating with a light cell. (2) Soon after they join, pairs may be forced apart by repeatedly expelling them from a micropipette. The mating types of members of such split pairs were always found to be complementary. (3) Attempts to induce "selfing" by exposing cells to filtrates from cells of other types have failed.

However, not all conjugations in *Oxytricha* involve cells of complementary mating types. Some cultures may self whenever conditions for mating are proper. For example, cultures of stock 24A, mating type III (see Table I) regularly contain about one per cent mating cells. The progeny of split pairs obtained from such type III cultures behave as the parental culture; each new culture will self, but is predominantly type III. Hence, it is possible that selfing pairs consist of cells of the same type induced to mate by circumstances as yet unknown. Alternatively, selfing cultures may consist of a small number of cells of an unstable mating type complementary to the predominant and more stable type, a situation which would result in selfing.

The remarkable mating behavior of *Oxytricha* prior to conjugation merits description despite similarity to the "Paarungsspiel" previously described for *Stylonychia,* a closely related genus, by Grell (1951). When cultures of complementary types are mixed the activity of the cells remains unchanged for about two hours. Thereafter, small groups of cells collect on the bottom of the culture dish and within each group the cells describe contiguous circles. Soon two such "dancing" cells meet at their anterior adoral surfaces and become relatively quiescent. The mates so joined may presently separate and find other partners or become more firmly united. If conjugation proceeds each cell is supported on the bottom of the dish by its posterior cirri, and the pair forms an obtuse angle. This angle is slowly reduced so that the cells finally adhere tightly along most of their lengths and conjugation is completed in this position. Mating behavior in *Euplotes* has been found to conform in general to the description given above (Kimball, personal communication; Siegel, unpublished). The complicated pattern of mating behavior observed in these organisms suggests that such cells bear a "sticky" stuff on localized surface areas which must be brought together in order to initiate conjugation. In contrast, other ciliates such as *Paramecium* form large clumps of mating cells; these animals are apparently sticky all over, initial unions are non-specific, and mating behavior is comparatively simple and direct.

Nine mating types are presently known, although relatively few stocks and hybrids from but two crosses have been studied as yet (Table I). Each type will conjugate with all of the other eight types but, with the exception of type III, will not self in unmixed controls. It seems quite likely that new stocks and other hybrids will provide additional mating types. Whether the species *O. bifaria* encompasses genetically isolated subspecies or "varieties" at least superficially comparable to those in *Paramecium aurelia* and *P. bursaria* remains to be discovered.

Some crosses (I × II, III × V, V × VII) yield high proportions of viable hybrids while the majority of the hybrids of other crosses (I × III, II × V) die. Hybrid mortality could be due either to strain differences or to the incompatibility of certain mating types *per se.* In contrast to these results, selfing is almost invariably followed by death, even in the case of cultures which can be successfully outcrossed. This fact suggests that lethal factors, responsible for mortality in both selfing and certain outcrosses, may be discovered in further genetic analyses of breeding relations.

Oxytricha exemplifies the ciliate life cycle first described in Maupas' (1888; 1889) monumental papers and intensively studied by Jennings (1944) in *Paramecium bursaria.* Starting as exconjugants, clones pass through stages of immaturity, adolescence, maturity, senescence, and finally die. Following the separation of conjugants, the new clones undergo periods of vegetative reproduction during which they cannot be induced to mate and hence are called "immature." Adolescence is marked by the ability of the new clone to conjugate with some, but not all, of the established mating types. Moreover, when these cultures can be induced to mate they yield relatively small numbers of conjugating cells. The stages of immaturity and adolescence may last for only a month or may persist for as long as two years. After further reproduction the clone acquires the capacity to mate readily with representatives of all the mating types, save those of its own type, and is now mature. A few hybrid cultures have passed from maturity to senescence and death, but most remain mature for at least two years. The persistence of each of these life cycle stages reflects a pattern of changing cell heredity or progressive differentiation under the control of genetic factors which are as yet unknown. In view of the various hypotheses for the general phenomenon of aging (Jennings, 1929; Sonneborn, 1954; 1955), it would be of interest to re-investigate this well-known problem, taking advantage of controlled conjugations between strains characterized by dissimilar life cycles.

The life cycles of certain ciliates may be greatly lengthened or perhaps eliminated entirely with the discovery of proper nutritive media and culture conditions. For example, clones of *Tetrahymena* do not normally pass through senescence and die off, although, parenthetically, they do undergo definite stages of sexual immaturity followed by adolescence and maturity. The following discussion pertains to other ciliates which show a more or less well-defined and complete life cycle. The important point for our purpose is that under conditions in which such a complete life cycle appears, fertilization has a rejuvenating effect. In these organisms senescence and death seem inevitable in the absence of nuclear reorganization, either uniparental autogamy (perhaps described as endomixis in some forms) or conjugation. If clones are aged, conjugation results in the death of most or all of the enconjugants (Jennings, 1944) and rejuvenescence occurs in only a few or none of the exautogamous progeny (Pierson, 1938; Sonneborn, 1954). Therefore, the periodic occurrence of some form of nuclear reorganization resulting in the formation of new clones would appear essential for the successful maintenance of such ciliate species. Moreover, since these processes may provide novel genotypes and genetic recombinations, they could be of some evolutionary consequence. The development of mating types must have been influenced by these fundamental factors. The significance of systems of binary mating types, as in *P. aurelia* and *P. caudatum,* and multiple mating types, as in *P. bursaria* and *Oxytricha,* will be considered in the next paragraphs.

All else being equal, species with a system of multiple mating types would be expected to conjugate more frequently than species with a system of binary mating types, for contacts between cells of complementary mating types would occur more often. The inadequacy of conjugation as the sole mechanism for nuclear reorganization in species with systems of binary mating types may have led to the development of a process for uniparental nuclear reorganization, namely, autogamy. Or the reverse may have occurred; species which failed to evolve autogamy developed compensatory systems of multiple mating types. At any rate, the two species of *Paramecium* for which binary mating types are known (*P. aurelia* and *P. caudatum*) pass through autogamy, whereas in those three species with multiple mating types (*P. bursaria, P. multimicronucleatum, P. trichum*) autogamy is extremely rare or lacking. Autogamy has not been reported to date in *Euplotes patella, Stylonychia putrina,* or *Oxytricha bifaria* despite intensive efforts, in some cases at least, to detect its occurrence. These species manifest multiple mating types. The available data thus agree with the interpretation of a causal relationship between the kind of mating system and autogamy. Clearly many more species must be investigated before this hypothesis can be adequately evaluated. For example, it would be important in this connection to discover mating systems for *Stylonychia pustulata* and *Paramecium polycaryum,* species in which autogamy has been described by Fermor (1913) and Diller (1954), respectively. On the other hand, one of the six known varieties of *P. bursaria* is enigmatic, for only two mating types have as yet been collected from natural sources. Furthermore, endomixis has been reported for certain cultures of *Oxytricha bifaria* by Kay (1946); massive conjugation epidemics could be induced in wild cultures and mating was regularly preceded by the appearance of a few scattered animals described as endomictics. Unfortunately, this worker was unable to demonstrate mating types among these and other cultures.

Finally, it should be emphasized that although ciliates such as *Tetrahymena* do not pass through stages of senescence and death under artificial laboratory conditions, they may complete the life cycle in natural habitats. If so, the interfertile clones of *Tetrahymena pyriformis* studied by Nanney and Caughey (1953), which form a pattern of multiple mating types and do not pass through autogamy, would lend support to this hypothesis. The fact that *Tetrahymena rostrata* undergoes autogamy (Corliss, 1952) invites an investigation of the mating reactions in that species.

An entirely different explanation for the alternative systems of mating types should be considered. Multiple mating types may occur among species whose natural populations are small, thereby enhancing the chances for cells to encounter suitable mates. On this hypothesis species with binary mating types would be expected to be relatively more populous. However, adequate population studies have not yet been made and the significance of mating systems remains a matter of speculation.

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SUMMARY

A study of *Oxytricha bifaria* revealed a system of nine interbreeding mating types. The mating behavior and life cycle of this organism are described. Two hypotheses which account for systems of multiple mating types in some ciliates and binary mating types in others are presented.

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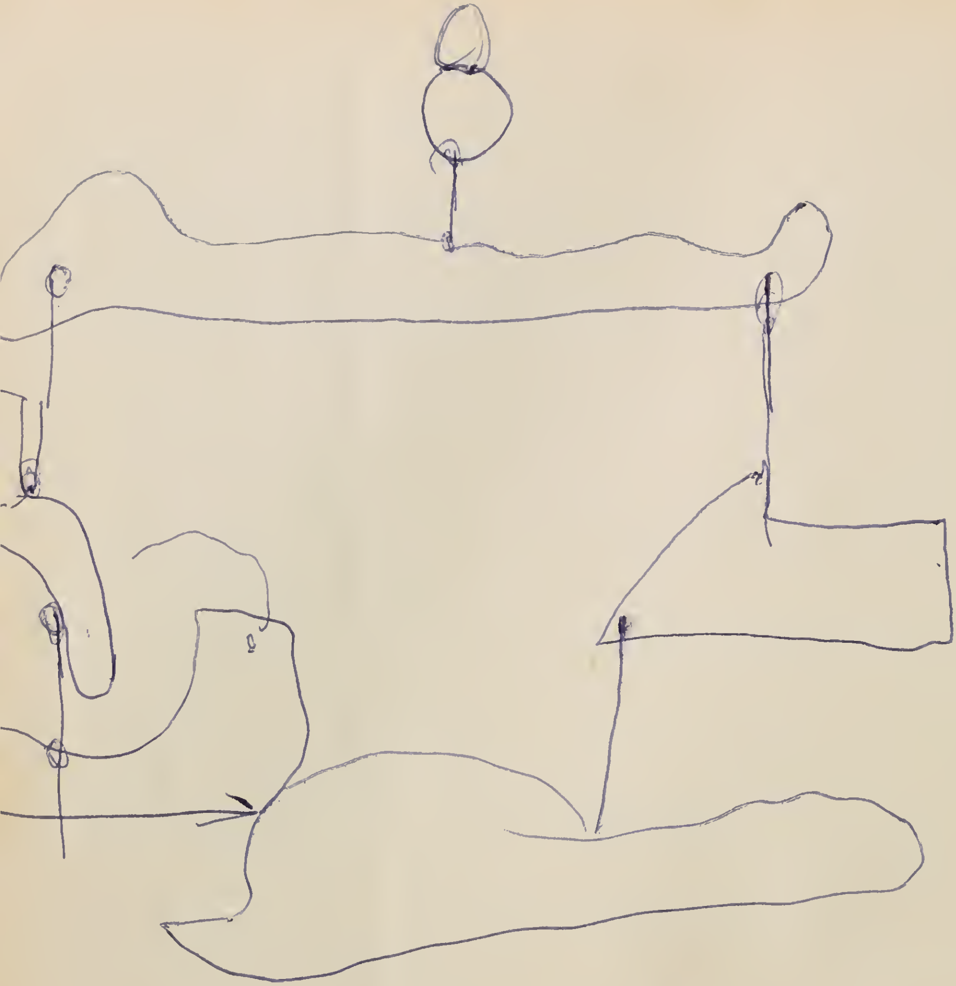

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